Exploring the Gut Microbiome in Chronic Kidney Disease

By
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ABSTRACT

Patients with chronic kidney disease (CKD) exhibit a disproportionate burden of cardiovascular disease and other comorbid conditions compared to the general population. It is speculated that this increased burden of comorbid conditions is partially driven by the systemic accumulation of gut-derived bacterial byproducts and the ensuing activation of innate immune pathways. As a result, researchers have directed their attention to the intestinal microbiome as a novel therapeutic target for reducing morbidity and mortality in CKD patients.

In this dissertation, I will first summarize the current state of knowledge regarding the complex interactions between the intestinal microbiota and gut barrier function, highlighting how intestinal bacteria and components of the intestinal barrier contribute to the maintenance of an immune tolerant environment. Next, I will review the existing literature regarding the contribution of intestinal dysbiosis to chronic disease states, including CKD. Lastly, I will review the findings from my own experiments focused on: (1) determining how CKD alters the metabolism of trimethylamine *N*-oxide (TMAO), a novel uremic toxin that correlates with cardiovascular risk in CKD, and (2) characterizing how CKD impacts the expression patterns and activity of intestinal and hepatic alkaline phosphatases, a group of enzymes involved in the detoxification bacterial endotoxins and maintenance of intestinal barrier function.

Acknowledgements

First, I must thank my mentor Dr. Jason Stubbs. Dr. Stubbs was not looking to mentor a graduate student until I came along. Times were tough in trying to develop a thesis project when I kept only obtaining negative data to share with him, yet I had his complete support along the way. I thoroughly enjoyed our meetings and getting the chance to learn about his perspective as a medical doctor on the clinical impact of each of the projects I worked on. Every interaction I had with Dr. Stubbs, I left a better presenter and communicator. I would be remiss if I did mention the important role Shiqin Zhang played in my training, especially early in my career. I vividly remember how upset I was after euthanizing my first mouse, but Shiqin was there to help me gather myself and clean up. A few months, or maybe even close to a year after this incident, she was always willing to inject and bleed my mice when I was too nervous. Lastly, my current projects would not have moved as quickly if it were not for Faith Omede. Faith took on experiments that I dreaded, and was always understanding when I gave him inaccurate instruction, resulting in him having to redo an assay.

For the last 5 years I have had my committee's unwavering support and attention as I presented a new project at each meeting I held. When I was finally able to show preliminary data of changes in intestinal alkaline phosphatase activity, everyone shared in my excitement. Dr. Katherine Swenson-Fields, with her office located just a floor below our lab, was constantly available to field any questions I had, and I left every interaction learning more than I set out to. Dr. Michele Pritchard's expertise was imperative as I began to explore changes in the liver in a kidney-centric lab space. Dr. Alan Yu was always there to challenge whether my data backed the conclusions I was

making. Lastly, Dr. Brenda Rongish was always eager to answer questions and make sure I progressed through the Anatomy requirements efficiently.

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CHAPTER 1: INTRODUCTION

Clinical Implications of Chronic Kidney Disease

A major function of the kidney is to maintain homeostasis by excreting excess metabolites and waste products from the blood. This is achieved by the coordinated action of approximately 1 million nephrons per kidney (1). Bulk filtration of the blood occurs at the glomerulus, a tuft of capillaries encapsulated by Bowman's capsule, while fine-tuning of the filtrate occurs throughout the remaining nephron segments to produce the final urine product. Kidney function is assessed by calculating the estimated glomerular filtration rate (eGFR) of a substance that is predominately filtered by the glomerulus and not further handled by the downstream nephron. Most often in the clinic, a patient's eGFR is calculated based on measurements of their serum and urine creatinine concentrations (2). Patients with normal kidney function have an eGFR of roughly 120 mL/min, resulting in 180 L of blood being filtered by the kidneys per day.

Common risk factors for the development of chronic kidney disease (CKD) include obesity, diabetes, metabolic syndrome, and hypertension; therefore, the increasing prevalence of Western diet consumption and a sedentary lifestyle among Americans has resulted in a growing number of individuals developing kidney disease in the United States (3). CKD is characterized by the progressive and irreversible decline in kidney function, and CKD severity is classified into five stages, largely based on eGFR values. At CKD stage 3 (an eGFR ~60 ml/min), a patient's glomerular filtration has declined by at least 50%, resulting in the accumulation of biological waste products that exhibit toxicity to a variety of organ systems. A patient with minimal residual kidney

function (<10%) is considered to have end-stage renal disease (ESRD) and must receive either chronic dialysis therapy or a kidney transplant in order to survive.

CKD patients are at an increased risk of developing numerous comorbid diseases, most notably, cardiovascular disease (CVD). Moreover, the increased risk of CVD observed in CKD patients persists after the adjustment for traditional risk factors, such as diabetes and hypertension (4). Researchers hypothesize that uremic toxins, metabolites that accumulate in the setting of decreased renal clearance, and a pro-inflammatory phenotype, both contribute to pathological changes that promote more aggressive CVD development in patients with impaired kidney function. While a subset of these uremic toxins are metabolites formed by the processing of dietary constituents by intestinal bacteria, others are pro-inflammatory bacterial byproducts that likely gain entry to the portal circulation through intestinal barrier defects.

The purpose of this dissertation is to examine how alterations to the intestinal microenvironment in CKD may contribute to the pathophysiology of comorbid conditions, such as CVD, that are highly prevalent in CKD patients.

Intestinal Microbiota

The intestinal microbiota is composed predominantly of bacteria belonging to *Firmicutes* and *Bacteroidetes* phyla, but other organisms such as achaea, viruses and fungi are also present. The exact composition of the intestinal microbiota is dependent on the anatomical location within the digestive tract. For example, due to the acidic environment, few microorganisms are found within the stomach, and the number of bacteria increases dramatically moving distally to the colon (5). Furthermore, host diet

can have a substantial impact on intestinal microbiota composition that is at least partially dependent on dietary protein and carbohydrate intake (6).

With nearly 1 trillion cells, culminating to 150 more genes than expressed by mammalian systems (7), the intestinal microbiota performs indispensable functions to maintain normal host physiology such as, metabolizing complex polysaccharides to produce short-chain fatty acids (SCFA) and synthesizing crucial vitamins (8,9). These microorganisms are imperative for both intestinal and immune system development. Germ free mice, void of intestinal microbiota, have gross morphological abnormalities of their intestine (10-12) and a naïve immune system that is unable to properly generate an immune response (13). Additionally, the importance of the intestinal microbiota in the developing intestine is highlighted by the 'hygiene hypothesis'. This hypothesis states that the increasing incidence of autoimmune diseases in developed counties is due to decreased exposure to bacteria in early life, resulting in improper education of the immune system (14).

Intestinal Barrier

The digestive tract is one of the most immunologically active organs, and due to the constant exposure to bacteria and other microorganisms within the digestive tract, the body has developed a complex barrier system to maintain a sterile systemic environment (Figure 1.1 A). The three layers of the intestine include the mucosa (luminal side), submucosa, and muscular layer. The mucosa is composed of intestinal epithelial cells (IEC) and the lamina propria. The architecture of the small intestine includes finger-like projections, villi, and crypts to increase surface area and promote

absorption of nutrients. The intestinal submucosa contains blood vessels and nerves, while the muscular layer is crucial for peristalsis.

As depicted in Figure 1.1 A, the mucus layer adjacent to the mucosa is composed of mucin proteins and acts as an initial physical barrier for microorganisms. Within the mucus layer, antimicrobial peptides (AMP), secreted by Paneth cells, and soluble IgA, secreted by plasma cells that reside in the lamina propria, maintain a relatively sterile environment adjacent to the intestinal epithelium. Next, tight-junction complexes between IEC do not allow for the paracellular transport of large molecule such as bacteria or bacterial byproducts (Figure 1.1 B). Lastly, cells of both the innate and adaptive immune systems reside within the lamina propria (Figure 1.1 C). As described above, terminally differentiated plasma cells secrete soluble IgA and T cell populations secrete various cytokines and factors. Other cells of the innate immune system that reside in the lamina propria include macrophages with phagocytic capabilities and dendritic cells with antigen-presenting capabilities. Commensal bacteria can promote the maintenance of the intestinal barrier, along with each component of the barrier regulating one another. As just one example, the overexpression of the antimicrobial peptide (AMP) results in a thicker mucus layer that bacteria cannot penetrate as readily (15).

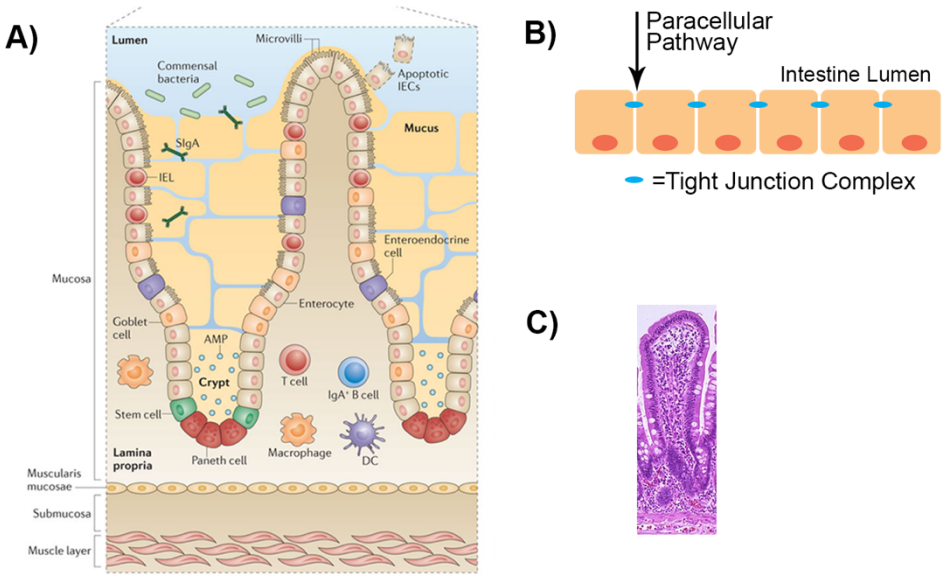

Figure 1.1 Schematic of the intestinal barrier. (A) The three layers of the intestine include the mucosa, submucosa, and muscle layer. The body has developed numerous mechanisms to maintain immune tolerance and prevent bacteria from entering into systemic circulation. The mucus layer, containing antimicrobial peptides (AMP) and soluble IgA (SIgA), is a relative sterile environment (16). **(B)** Tight junction complexes between intestinal epithelial cells prevent the paracellular transport of bacteria or bacterial endotoxins from entering into the circulation. **(C)** The lamina propria just underneath the intestinal epithelial cells contains cells from the innate and adaptive immune systems.

Role of Intestine in Disease

As described above, the intestinal barrier and immune system have developed a complex network to maintain normal physiologic homeostasis and prevent exposure to the numerous inflammatory stimuli that reside in the gut lumen. If mechanisms are lost to maintain immune tolerance, sporadic disease can occur as observed in the *IL10*$^{-/-}$ mouse. IL10 is an anti-inflammatory cytokine involved in maintaining immune tolerance within the intestine, and when void of this cytokine, sporadic inflammatory bowel disease develops. Interestingly, when the *IL10*$^{-/-}$ mouse lacks intestinal bacteria, no disease of the intestine occurs (17). Likewise, genetic mutations linked to inflammatory bowel disease, including alterations to NOD2 and JAK-STAT signaling, lead to defects in bacterial sensing and subsequent immune responses (18).

Transgenic overexpression of myosin light-chain kinase, which results in the loss of tight junction integrity and increased intestinal permeability, is not sufficient to cause intestinal disease, despite observing a mucosal immune response in this setting (19). In numerous chronic diseases, it is believed that subtle shifts in intestinal microbiota composition and alterations to barrier function do not lead to an outright inflammatory phenotype within the intestine, but rather, these changes provide an environment that is more amenable to translocation of luminal contents into the systemic circulation to trigger pro-inflammatory cascades.

Bacterial Endotoxin: As will be described below, a majority of chronic diseases are characterized by a pro-inflammatory phenotype that may be due to the translocation of bacteria and bacterial endotoxins into systemic circulation. Endotoxins are components of bacteria that accumulate due to bacterial cell death and can elicit an

immune response through binding of pattern recognition receptors of the innate immune system. Lipopolysaccharide (LPS) is the most extensively studied endotoxin, and is a component of the bacterial wall of gram-negative bacteria. The structure of LPS includes an O-antigen, a polysaccharide core, and a lipid A component. The O-antigen is the most variable region of LPS and is used by different bacterial species to evade immune recognition. By contrast, the lipid A motif is imperative for binding to the LPS receptor, toll-like receptor 4 (TLR4), to stimulate an inflammatory response (20,21). Increased systemic LPS is most notably associated with metabolic syndrome and associated conditions (22).

Aging: Increased intestinal permeability is an evolutionarily conserved hallmark of aging observed in *Drosophila*, *C. elegans*, mice, and humans. In *Drosophila*, increased intestinal permeability was a better predictor of mortality than chronological age (23). Furthermore, in these invertebrate models, shifts in intestinal microbiota composition occurred before increased intestinal permeability (24). In mammalian systems, an aged intestine begins secreting more pro-inflammatory cytokines (25), many of which have been shown to disrupt intestinal epithelium tight junction complexes (26,27). While there have been no gross abnormalities observed in the mucus layer lining of an aged intestine, Paneth cell numbers decline and are associated with reduced AMP secretion (28), and increased bacterial adhesion to the mucus layer (29). Lastly, investigators have observed an age-related decline in antigen presenting cells in the lamina propria, resulting in impaired T cell priming (30). Chronic diseases share many similarities to the aging process, and a common link appears to be increased

intestinal permeability. It may be possible that treatments targeting the gut could be a therapeutic tool utilized for several of these conditions

Western Diet: Diet is a well-recognized risk factor for the development of chronic diseases. A Western diet, characterized by consumption of highly processed foods with high saturated fat content, promotes intestinal inflammation (31) and increased intestinal permeability (32,33) resulting in increased serum LPS levels. This disease state, termed metabolic endotoxemia, leads to insulin insensitivity, fatty liver disease, and increased risk of CVD development. Additionally, Lee *et. al.* described a decrease in Paneth cell and goblet cell number with consumption of a high-fat diet (33). Moreover, as diet and microbiota composition are intimately linked, these anatomical changes may be due to changes in gut bacterial species with consumption of a high fat diet (34). It is noteworthy that germ free mice do not become obese when fed a high fat diet (35,36).

Liver Disease: Gut luminal content absorbed into the bloodstream enters into the portal vein and is handled first by the liver before continuing on to the systemic circulation. Due to this intimate relationship, it is understandable that patients with liver disease would exhibit intestinal dysbiosis. Alcoholic liver disease is associated with small intestinal bacterial overgrowth, reduced colonic bacteria (37), and low-grade endotoxemia (38,39). One hypothesis among researchers is that acetaldehyde, a toxic metabolite generated by the metabolism of ethanol, drives shifts in the intestinal microbiota and increases intestinal permeability by disrupting the tight-junction integrity between IEC (40). In addition to decreased tight-junction protein expression, a thickened mucus layer is observed in mice and humans after chronic alcohol exposure

(41). Intestinal changes are also observed in non-alcoholic liver disease (42) and cholestasis induced by bile duct ligation (43).

Diabetes: In addition to diseases associated with life-style factors, alterations to the intestinal microbiota and immune system have been implicated in autoimmune diseases; such as, type 1 diabetes (T1D). Early studies in T1D rodent models revealed that before the onset of insulin resistance, there is an increase in intestinal permeability, increased intestinal immune infiltration, and a decline in natural killer cell number and function within the intestine (44-46). A clinical study explored the serum levels of zonulin, a protein that negatively regulates intestinal permeability, in patients with T1D. The authors of this study concluded that there was an upregulation of zonulin in patients with T1D compared to healthy controls. Furthermore, increased serum zonulin levels were observed in a subset of patients in the pre-type 1 diabetes phase (47). In addition to local immunity, T1D researchers have explored alterations to the enteric nervous system. Briefly, T1D results in the loss of neuron number and overall motor function of the gut (48).

Gut Microbiome Changes in CKD

Studies conducted over 50 years ago examining the possible role of the microbiota in kidney disease elucidated that post-nephrectomy, germ-free rats survived significantly longer than conventionally raised rats (49). More recently, the presence of systemic bacteria and bacterial endotoxins were confirmed in uremic animal models (50-53) and in ESRD patients; these bacterial endotoxins were predictive of an enhanced inflammatory phenotype (54). Clinical studies, utilizing non-invasive measurements of intestinal permeability (urinary recovery of polyethylene glycols [PEG]

or plasma D-lactate measurements), suggest that ESRD patients may have increased intestinal permeability compared to healthy controls (55).

To date, most of the studies investigating the intestinal environment in CKD have focused solely on shifts in the intestinal microbiota (56) and expression of tight-junction proteins in CKD animal models. For example, in the 5/6 nephrectomy model, rodents with reduced nephron mass exhibit decreased expression of the tight junction proteins, occludin, zona occludin-1 (ZO-1), and claudin-1, in whole tissue lysates of the stomach, jejunum, ileum, and colon (57,58). Additional evidence from *in vitro* studies demonstrated that serum from ESRD patients was capable of decreasing epithelial resistance and accompanying tight junction protein expression in T84 cells, a human enterocyte cell line (59). In a separate study utilizing these same T84 cells, applying urea alone resulted in diminished tight junction protein expression. Moreover, protein expression was further reduced when cells were exposed to urea in conjunction with urease, a bacterial enzyme capable of converting urea to ammonia. Based on these results, authors of this study hypothesized that an accumulation of urea within the digestive tract of patients and animals with CKD is being converted to ammonia that increases the pH of the intestinal tract, which in turn, disrupts tight junction protein complexes (60). There are certainly many other bacterial metabolites/toxins or local environmental factors that may contribute to diminished intestinal epithelial tight junction integrity in CKD (61).

As described above, many uremic toxins that accumulate in CKD and are thought to have organ toxicity are derived from intestinal microbes. The two most widely studied uremic toxins of intestinal origin are the protein-bound solutes indoxyl sulfate

and p-cresyl sulfate. Indoxyl sulfate is derived from the amino acid tryptophan (62) and is primarily associated with kidney fibrosis (63), and vascular dysfunction (64,65). P-cresyl sulfate, produced by bacterial metabolism of tyrosine and phenylalanine, is associated with adverse CVD outcomes and all-cause mortality (66,67). Other potential uremic toxins of intestinal origin that have been studied less extensively include phenylacetylglutamine (68), guanidine (69), and trimethylamine *N*-oxide (70). Lastly, metabolic shifts in the intestinal microbiota may result in increased capacity of the intestinal microbiota to produce uremic toxins and decreased capacity to produce SCFA (71-73). The changes observed in patients with CKD are summarized in Figure 1.2.

Treatments Targeting the Gut Microbiome in CKD

Clinical strategies seeking to reduce circulating levels of uremic toxins and inflammation in CKD patients include protein restriction and renal replacement strategies; thus far, both have proved ineffective. In the past two decades, researchers have turned to therapies targeting the intestinal microbiota and intestinal environment. Therapies that have gained attention include oral adsorbents and probiotics. Unfortunately, as of now, no therapy has shown enough promise and safety to gain FDA approval.

Oral adsorbents bind and promote the fecal excretion of uremic toxins. The most extensively studied adsorbent, is activated charcoal AST-120. This therapy restored colonic tight-junction protein expression in an adenine-induced CKD rat model (58), reduced a kidney fibrotic phenotype in a nephrectomized rat model of CKD (74), and even reduced the incidence of cardiac fibrosis in a rat model of CKD through attenuation of oxidative stress (75). AST-120 therapy reduced serum concentrations of

indoxyl-sulfate in uremic animals and in a phase II clinical trial in humans (58,74,76). However, in the Evaluating Prevention of Progression in CKD (EPPIC) study, a phase III trial investigating the effect of AST-120 on CKD progression, there was no obvious impact of this therapy on CKD progression; thus, this drug has failed to gain FDA approval. A major limitation to this study was that the placebo-treated group had slower than expected disease progression, making it difficult to observe any significant differences with AST-120 treatment (77). It is noteworthy, that AST-120 therapy is approved and extensively used in Japan as a possible mechanism for delaying the time to renal replacement therapy. Reported adverse events with AST-120 therapy include constipation, appetite loss, and nausea (78).

Another strategy to reduce uremic toxins and inflammation is the administration of probiotics, live bacteria that confer a health benefit to the host. Several bacterial strains can be administered simultaneously to achieve multiple endpoints of interest. Probiotics can confer benefit to the host through several mechanisms, including competing for environmental resources with other bacterial strains to reduce the growth of pathologic bacteria (79), or by affecting host biology through actions such as stimulating the production of mucins (80), enhancing tight-junction integrity (81), promoting the production of antimicrobial peptides (82), or reducing the pH of the digestive tract (83,84).

A promising use for probiotic therapy in CKD would be the administration of bacteria that could metabolize specific uremic toxins or target metabolic pathways involved in uremic toxin production. This could be achieved through administering several bacterial strains simultaneously, or genetic engineering to produce bacteria that

express enzymes of interest. While strategies such as microencapsulation of probiotics have significantly improved the effective delivery of these therapies to patients (85,86), special consideration will need to be taken into account when designing probiotic therapy for treatment of CKD patients, since this population exhibits unique biochemical parameters (i.e. chronic acidosis) and altered intestinal physiology (i.e. decreased transit time) that may affect the successful colonization of probiotic species.

Gaps in Knowledge

In CKD, researchers believe that uremia results in shifts of the intestinal microbiota composition leading to increased capacity to generate uremic toxins. Shifts in intestinal microbiota composition are accompanied by increased intestinal permeability that may play a role in the endotoxemia and pro-inflammatory environment observed in patients with CKD. Additionally, diminished renal clearance and systemic alterations play a major role in the accumulation of gut-derived uremic toxins and inflammatory cytokines further exacerbating these phenotypes in patients with CKD (Figure 1.2). As compared to other disease states described above, studies are sparse as to whether there truly is a functional defect in the intestinal barrier in CKD mouse models. It is difficult to determine whether these phenotypes are due to changes in the intestinal environment or reduced renal clearance; however, the gut serves as a promising long-term therapeutic target that can be manipulated with minimal anticipated side effects.

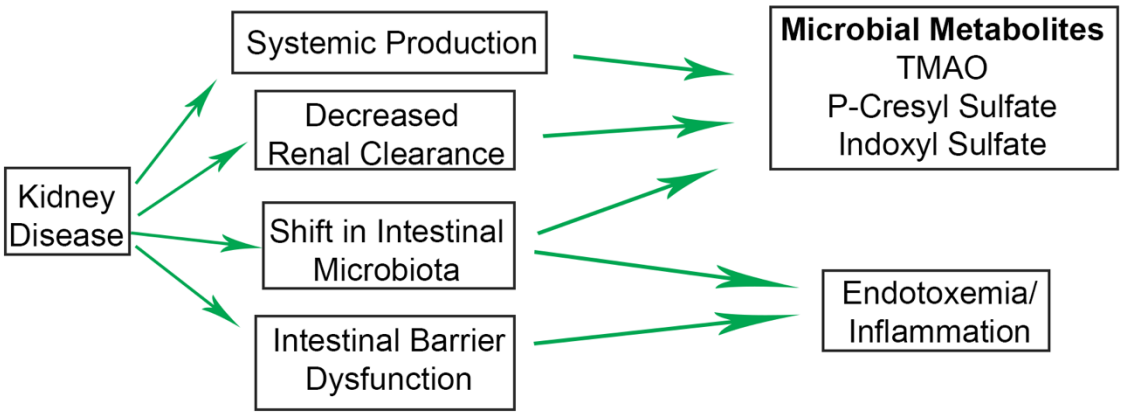

Figure 1.2 Kidney disease results in the accumulation of microbial metabolites and endotoxemia. In addition to decreased renal clearance, CKD patients exhibit systemic and intestinal changes that results in the accumulation of microbial metabolites and endotoxemia.

To date, research in CKD has only focused on the composition of the intestinal microbiota and expression of intestinal tight junction proteins. Furthermore, studies have provided conflicting results as to whether there truly is decreased intestinal tight junction protein expression. Contrary to other published reports in CKD models, Anderson *et. al.* observed by electron microscopy that there appears to be no change in intestinal tight-junction ultrastructure in the *Col4α3*$^{-/-}$ CKD mouse model (50). Lastly, the mechanism leading to intestinal dysbiosis in CKD has not been determined. Urea may be an initial driver, but the role of other uremic toxins and LPS, which disrupts tight-junction protein expression (61), has yet to be explored.

To successfully design therapies targeting intestinal environment in CKD patients, a full examination of the intestinal microbiota and intestinal barrier is warranted. First, technology is to the point where metagenomic and metatranscriptomic sequencing of the intestinal microbiota is more easily performed. These types of analyses provide valuable information about shifts in the metabolic pathways rather than shifts in relative microbial abundance that is the focus of 16S rRNA sequencing. As the technology progresses and price decreases, it may become possible to develop personalized therapies targeting metabolic pathways altered in each individual patient with CKD.

In regard to the intestinal barrier, studies first determining changes in each component of the barrier are warranted. This would include characterizing the composition of the mucus layer, determining whether there is diminished AMP production, and better characterizing the intestinal immune state in CKD animals. In addition to the intestinal barrier and immune system, studies have yet to explore the

effect of kidney disease on components of the enteric nervous system, especially as a common symptom in CKD patients is diminished gut motility (87,88).

In this dissertation, I will highlight my work to: (1) investigate how CKD alters the metabolism of trimethylamine *N*-oxide (TMAO), a novel uremic toxin thought to contribute to cardiovascular disease in CKD, and (2) determine if alterations in intestinal alkaline phosphatase expression and activity may contribute to impaired intestinal barrier function and microbial population shifts in CKD animals.

Chapter 2: Chronic Kidney Disease Increases Flavin Monooxygenase Activity and Circulating Trimethylamine N-Oxide Concentrations in Mice

INTRODUCTION

Patients with chronic kidney disease (CKD) exhibit a substantial burden of cardiovascular disease (CVD) that is disproportionate to that observed in patients with comparable comorbidities and normal kidney function (89-91). It is clear that this increased burden of CVD in CKD patients is not solely driven by a higher prevalence of traditional risk factors for CVD development, such as diabetes and hypertension (4). Emerging evidence suggests that a number of metabolic byproducts produced by intestinal bacteria may serve as cardiovascular toxins and non-traditional risk factors for accelerated CVD progression in CKD (92).

Through an unbiased metabolomics approach, trimethylamine *N*-oxide (TMAO) and its precursors, L-carnitine and choline, were found to correlate with CVD outcomes in patients with normal kidney function. The bacterial metabolism of dietary L-carnitine and choline produces the gas trimethylamine (TMA), which is rapidly absorbed into systemic circulation and oxidized by the hepatic flavin monooxygenase (FMO) 3 enzyme. Antibiotic treatment in both rodents and humans resulted in diminished TMAO production after choline or L-carnitine challenge, further proving the necessity of the microbiota in its production (93,94).

The direct stimulation of CVD outcomes by TMAO was elucidated in various rodent models. Most notably, high serum TMAO levels resulted in increased atherosclerotic plaque burden in the *ApoE*$^{-/-}$ mouse, a model predisposed to atherosclerosis development, while antibiotic therapy to lower serum TMAO attenuated

disease progression (93,95). *In vitro* studies demonstrated that TMAO can also directly induce inflammation in vascular endothelial cells (96). In addition to atherosclerosis, TMAO is implicated in the development of cardiac fibrosis (97) and platelet hyperactivity (98).

CKD patients exhibit serum TMAO concentrations that far exceed those observed in patients with normal kidney function, with serum concentrations often 30 to 100-fold higher in patients with end-stage renal disease (ESRD) (70,99,100). Existing data support that TMAO clearance from the bloodstream occurs primarily through urinary excretion (101). However, formal studies investigating the mechanisms responsible for TMAO accumulation in CKD are lacking. In the current study, we utilized CKD mice to investigate how decreases in kidney function alter both renal excretion and hepatic production of TMAO.

METHODS

Animal Model and Urine Collection

All mice were maintained in accordance with recommendations in the “Guide for Care and Use of Laboratory Animals,” from the Institute on Laboratory Animal Resources, National Research Council (National Academy Press, 1996), and all animal protocols were reviewed and approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee prior to the commencement of this research. Kidney disease was induced by feeding C57BL/6J mice that were bred at the University of Kansas Medical Center a diet containing 0.2% adenine (Harlan Teklad, Madison, WI) starting at 6 weeks of age. Due to differences in kidney disease progression between sexes consuming an adenine diet, females were maintained on

the diet for 12 weeks, whereas males were provided the diet for 10 weeks (102). At the time of sacrifice, mice were placed in metabolic cages to obtain a fasting urine collection (12 hours). Prior to this fasting urine collection, mice were housed in the metabolic cages eight hours per day for three days for environmental acclimation and provided a 1% agar diet to promote hydration. At sacrifice, harvested tissue was either fixed for histology or snap frozen and stored at -80°C until further processing.

Serum and Urine Biochemistries

Serum blood urea nitrogen (BUN) and creatinine was measured using an Integra 400 plus bioanalyzer (Roche Diagnostics, Indianapolis, IN). Urine creatinine was measured by colorimetric assay (Caymen Chemical, Ann Arbor, MI), relying on the Jaffe reaction. Serum and urine TMAO was quantified by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as previously reported (103). Renal clearance and fractional excretion (FE) of TMAO was calculated using Equation 1 and Equation 2, respectively.

Eq 1:	$Renal\ Clearance\ \left(\frac{\mu L}{minute}\right) = \frac{urine\ [TMAO]\ (\mu M) \times urine\ flow\ (\mu L/min)}{serum\ [TMAO]\ (\mu M)}$
Eq 2:	$FE\ TMAO\ (\%) = 100 \times \frac{urine\ [TMAO]\ (\mu M) \times serum\ [creatinine](\frac{mg}{dL})}{serum\ [TMAO]\ (\mu M) \times urine\ [creatinine](\frac{mg}{dL})}$

Kidney Histology

Kidneys were fixed in 4% PFA for 24 hours and after paraffin embedding, 5 μm sections were obtained. Mid-sagittal regions of the kidneys were stained by the period-acid Schiff (PAS) method (Sigma, St. Louis, MO).

Assessment of Liver FMO3 Expression and Activity

For quantitative real time-PCR assessment of liver FMO3 gene expression, total RNA was isolated following homogenization of liver tissue using TRI-Reagent (Molecular Research Center, Cincinnati, OH). First strand cDNA was synthesized from 1 μg RNA using iScript (BioRad, Hercules, CA). PCR reactions contained 40 ng cDNA, 150 nM of each primer, and 1X iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) in 20 μl. The threshold cycle (Ct) of each gene product was normalized to the Ct for HPRT and gene expression was calculated using the Pfaffl Method (104). Primer sequences used were: FMO3-F:5'- CTCAGGCTGTGACATTGCTG -3', FMO3-R:5'CGACTCATCACCCAA GAACC -3'; HPRT-F:5'-TGATAGATCCATTCCTATGACTGTAGA -3', HPRT-R:5'AAG ACATTCTTTCCAGTTAAAGTTGAG -3'.

Hepatic FMO activity was assessed by quantifying FMO mediated formation of TMAO from TMA in liver microsomes. Microsomes were isolated from frozen liver tissue by differential ultra-centrifugation, as previously described with slight modification (105). Briefly, mouse livers from the same group were pooled together in sets of three and homogenized in ice-cold buffer (50 mM Tris-HCl buffer, 150 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol and 20% glycerol, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.113 mM butylated hydroxytoluene as protease inhibitors. The homogenate was centrifuged at 20,000*g* for 30 min at 4°C. The supernatant was then centrifuged at 140,000*g* for 30 min at 4°C. The microsomal pellet was re-suspended in 0.154 M KCl and was then centrifuged at 140,000g for 30 min at 4°C. The microsomal pellet was then finally re-suspended in 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose. Protein concentrations were determined using a Bio-Rad protein assay (Bio-

Rad Laboratories, Hercules, CA), and incubations were conducted for each pooled group using 0.75 mg/mL of microsomal protein in 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and 1 mM NADPH to assess FMO-mediated conversion of TMA to TMAO. Prior to adding the substrates, the microsomal incubations in the presence of NADPH were pre-warmed for 3 minutes at 37°C. Reactions were started by the addition of 3 µL of various concentrations of trimethylamine-HCl (5, 10, 25, 50, 100, 200, 500, 1000, 2500, and 10000 µM) and incubated for 60 min at 37°C. Each concentration of substrate was conducted in triplicate. The final reaction volume was 300 µL. Experimental controls omitting substrate and NADPH were included in each experiment. Incubations were stopped by adding 300 µL ice-cold MeOH. Final TMAO concentrations were quantified as described above.

Statistical Analysis

Comparisons between non-CKD and CKD animals were evaluated by Student's t-test, or Mann-Whitney test for data exhibiting a non-normal distribution. TMAO formation rates were used to calculate Michaelis-Menten enzyme kinetic parameters for FMO function. Calculations were performed using Prism 7 (GraphPad Software, San Diego, CA) and presented as mean ± SD. Parameters were compared by extra sum-of-squares F tests.

RESULTS

Assessment of kidney function and serum TMAO in CKD and control mice

Consumption of a 0.2% adenine diet led to substantial kidney injury in both female and male mice, as demonstrated by kidney histology revealing tubular injury and dilation, along with a marked infiltration of immune cells (Figure 2.1 A). Additionally,

CKD mice exhibited elevated serum blood urea nitrogen (BUN) concentrations (Figure 2.1 B; Females: CKD 117.2 ± 35.3 mg/dL vs non-CKD 26.1 ± 4.9 mg/dL, P<0.001; Males: CKD 120.5 ± 32.5 mg/dL vs non-CKD 28.5 ± 3.1 mg/dL, P<0.001). At baseline, there was a slight elevation in serum TMAO in female mice compared to male mice (Figure 2.1 C; Females 6.9 ± 6.1 μM vs Males 1.0 ± 0.5 μM, P=0.07). Serum TMAO concentrations in all CKD mice were considerably elevated compared to non-CKD controls (Figure 2.1 C; Females: CKD 29.4 ± 32.1 μM vs non-CKD 6.9 ± 6.1 μM, P<0.05; Males: CKD 18.5 ± 13.1 μM vs non-CKD 1.0 ± 0.5 μM, P<0.001).

Urinary Excretion of TMAO

There was a sex difference in the renal clearance of TMAO in non-CKD mice (Females 90.4 ± 78.1 μL/min vs Males 260.4 ± 134.5 μL/min, P<0.05). In both female and male mice, CKD resulted in a substantial decrease in the renal clearance of TMAO (Figure 2.2; Females: CKD 5.2 ± 3.8 μL/min vs non-CKD 90.4 ± 78.1 μL/min, P<0.01; Males: CKD 10.4 ± 8.1 μL/min vs non-CKD 260.4 ± 134.5 μL/min; P<0.001). In addition to decreased renal clearance, there was a decrease in the FE TMAO in both sexes with kidney injury (Figure 2.3; Females: CKD 52.5 ± 19.6% vs non-CKD 178.3 ± 157.3%; P<0.05; Males: CKD 64.8 ± 21.2% vs Non-CKD 240.9 ± 158.9%, P<0.01).

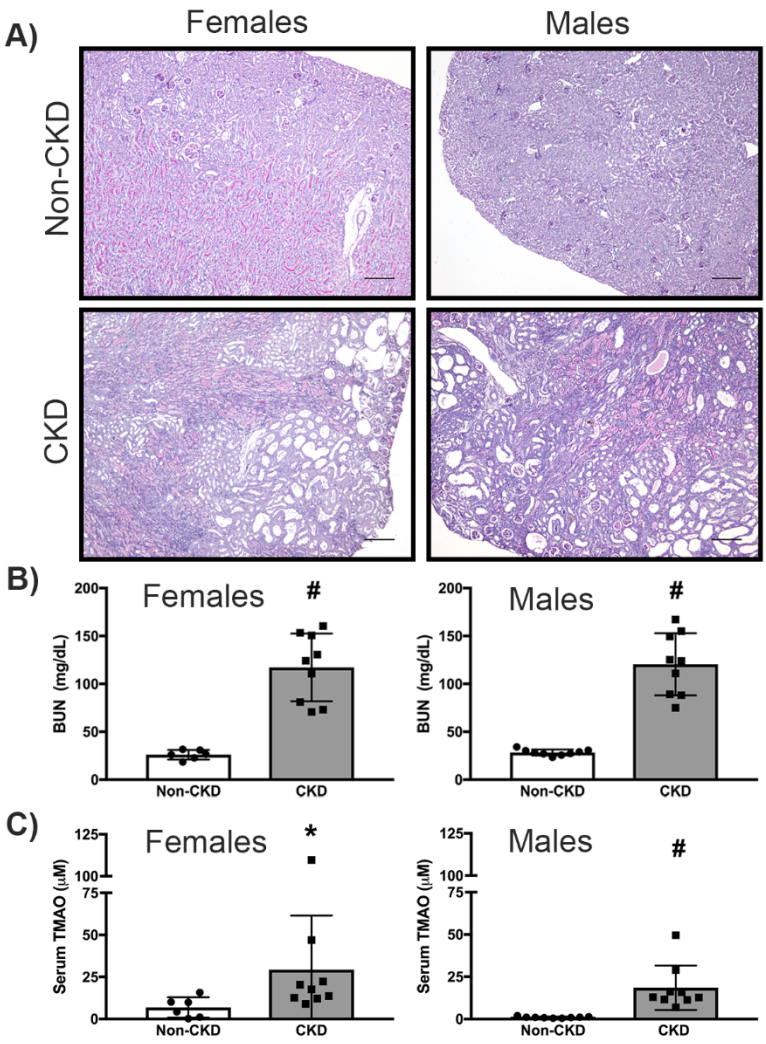

Figure 2.1 Adenine consumption led to impaired kidney function and an increase in serum TMAO concentrations. Chronic kidney disease (CKD) was induced by consumption of a diet containing 0.2% adenine, as described in the Methods section. **(A)** PAS staining of mid-sagittal kidney sections revealed intense immune infiltration and tubular dilation in both female and male CKD mice (scale bar=200 μm, 40x magnification) The induction of CKD was accompanied by a rise in **(B)** serum BUN, and **(C)** serum TMAO. * P<0.05, #P<0.001 vs non-CKD controls

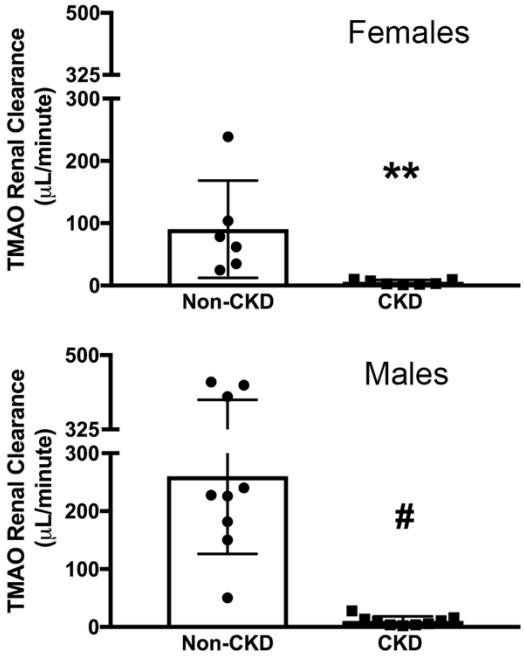

Figure 2.2 Kidney damage resulted in decreased renal clearance of TMAO. In both female and male CKD mice, there was a substantial decrease in the renal clearance of TMAO compared to non-CKD mice. **P<0.01, #P<0.001 vs non-CKD controls.

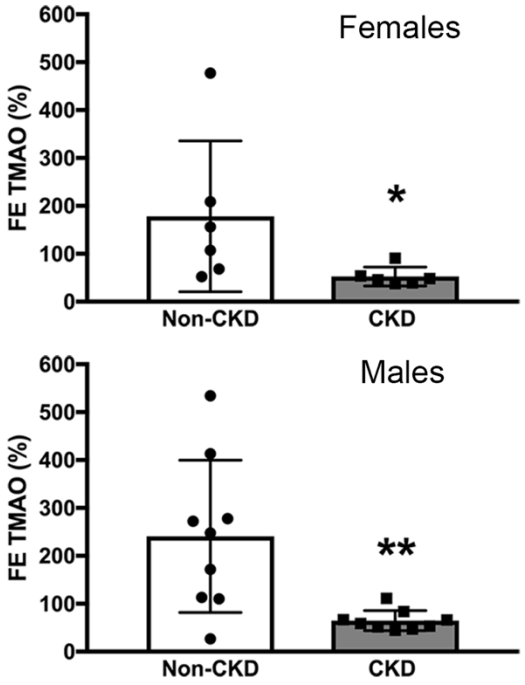

Figure 2.3 Impaired kidney function resulted in a decrease in fractional excretion of TMAO. The fractional excretion of TMAO (FE TMAO) in both female and male non-CKD mice was greater than 100%, suggesting a significant contribution of tubular secretion to renal clearance of TMAO in non-CKD animals. FE TMAO in the CKD animals was markedly decreased compared to controls. *P<0.05, **P<0.01 vs non-CKD controls

Liver FMO3 Gene Expression and Activity

Quantitative PCR was performed on liver tissue to assess the impact of CKD on hepatic *FMO3* gene expression. With chronic kidney injury, there was a 13-fold increase in FMO3 gene expression in females (Figure 2.4 A; $P<0.01$) and an 18-fold increase in males (Figure 2.4 A; $P<0.001$). To validate the functional significance of these gene expression changes with those observed in CKD mice, we performed *ex vivo* studies on hepatic FMO activity (Figure 2.4 B). At baseline, wild-type (non-CKD) females exhibited significantly higher FMO activity compared to males (Females 1838.0 ± 69.9 pmol/mg/min vs Males 1238.0 ± 26.0 pmol/mg/min; $P<0.001$). Similar to our observations with FMO3 gene expression, CKD mice exhibited enhanced hepatic FMO activity in both sexes (Figure 2.4 B; Females: CKD 2099.0 $\pm$ 36.4 pmol/mg/min vs non-CKD 1838.0 $\pm$ 69.9 pmol/mg/min, $P<0.001$; Males: CKD 2188.0 $\pm$ 46.1 pmol/mg/min vs non-CKD 1238.0 $\pm$ 26.0 pmol/mg/min, $P<0.001$).

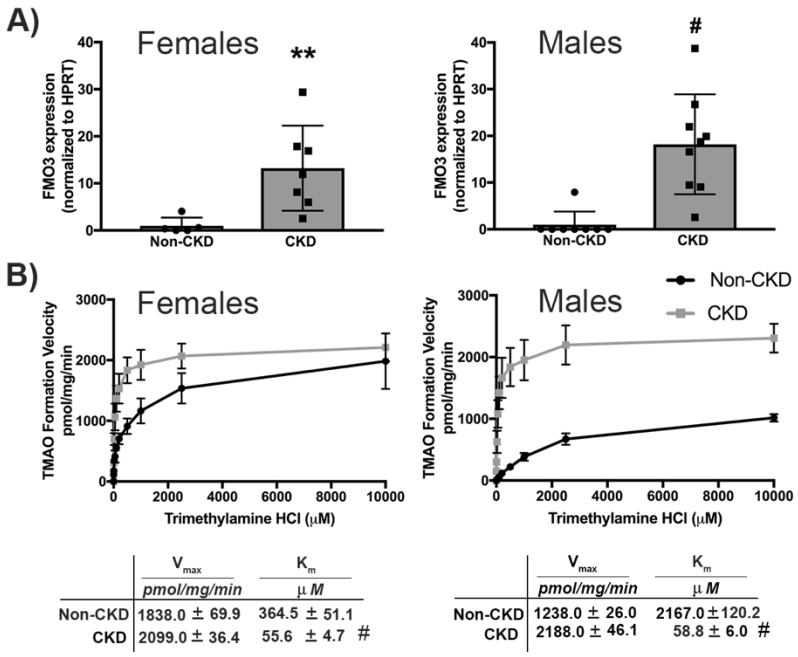

Figure 2.4 Increased hepatic FMO3 gene expression and activity in response to experimental CKD. (A) Quantitative-PCR analysis revealed an increase in liver FMO3 gene expression. **(B)** *Ex vivo* assessment of hepatic FMO activity from CKD and non-CKD mice. **P<0.01, #P<0.001 vs non-CKD controls

DISCUSSION

Based on data from both human and animal studies, TMAO is independently linked to atherosclerosis formation and CVD progression (93-95). Additionally, these investigations demonstrated a substantial elevation of circulating TMAO concentrations in patients with impaired kidney function; thus, TMAO may be an example of a non-traditional CVD risk factor that is of particular importance in this setting (70,106). While it has been widely speculated that increased increments in circulating TMAO in CKD primarily result from decreased urinary clearance of this compound, no formal studies have been conducted to rigorously test this hypothesis. Therefore, the current study was conducted to test how alterations to both urinary excretion and hepatic formation of TMAO may be altered in CKD using translational science approaches in mice.

Our first notable observation was discrepant serum TMAO concentrations between female and male wild-type (non-CKD) mice (Figure 2.1 C). While the difference in TMAO concentrations between healthy female and male mice was not statistically significant, mean TMAO concentrations were approximately 6-fold higher in the non-CKD females. Interestingly, female wild-type animals exhibited both decreased renal clearance and increased FMO activity compared to wild-type males (Figure 2.4). The observed difference in TMAO formation between sexes could be explained by the established function of testosterone serving as a negative regulator of FMO3 expression (107). As for the apparent sex difference in renal clearance of TMAO, studies evaluating tubular transport in rat kidneys have previously demonstrated a decrease in organic cation transporter (OCT)-2 expression in female animals (108). Since OCT-1 and OCT-2 are believed to be the primary regulators of TMAO secretion by tubular epithelial cells

(109), it is plausible that decreased renal TMAO clearance in wild-type females is a direct result of lower transporter abundance.

Similar to humans with CKD, mice with adenine-induced CKD exhibit substantially elevated circulating concentrations of TMAO. As anticipated, renal clearance of TMAO in CKD mice of both sexes is dramatically decreased (Figure 2.2). As depicted in Figure 2.3, the FE TMAO in the setting of normal kidney function is well above 100%, indicating that tubular secretion plays a predominant role in urinary elimination of this compound. Prior studies on tubular transport mechanisms in rats with adenine-induced CKD have found a dramatic decrease in tubular expression of both OCT-1 and OCT-2 (110), the suspected regulators of TMAO transport by renal tubules. Thus, we hypothesize that both a decrease in glomerular filtration and altered tubular secretion of TMAO actively contribute to its systemic accumulation in CKD.

Perhaps the most noteworthy finding of our current investigation was an increase in hepatic FMO3 gene expression and FMO activity in both male and female CKD animals compared to non-CKD controls (Figure 2.4). Increased hepatic FMO activity has previously been observed in streptozotocin induced diabetes model (111). To our knowledge, this is the first description of an upregulation of FMO3 activity in CKD models. It is unclear how and why FMO3 function would be altered in the setting of impaired kidney function; however, it is possible that this induction of FMO3 function by CKD could represent a compensatory mechanism to counteract the negative effects of uremia on normal physiological process. To this end, in fish, high concentrations of urea are thought to stimulate FMO3 activity leading to increased TMAO concentrations (112). Moreover, in the *in vitro* setting, TMAO has been observed to counteract the protein-

denaturing effect of urea (113,114). Based on this previously described beneficial function of TMAO, it is plausible that increases in urea, or associated uremic toxins, may stimulate hepatic FMO3 activity in an effort to counteract some of the detrimental effects of uremia. In turn, chronic exposure to high circulating concentrations of TMAO may have the unintended secondary outcome of promoting cardiovascular toxicity.

While studies support a correlation between circulating TMAO concentrations and CVD risk in CKD patients, no studies have confirmed whether lowering TMAO can attenuate CVD progression in this population. If future studies suggest clinical benefit of lowering TMAO concentrations in CKD patients, then it will be important to develop therapeutic strategies to target TMAO production or increase TMAO clearance from the circulation. It seems plausible that dietary interventions to lower the ingestion of TMAO and its precursors would likely alone be insufficient to dramatically lower the high concentrations of TMAO observed in CKD patients. Additionally, in the case of patients with ESRD, dialysis appears to offer only a transient lowering, with marginal long-term effect, on circulating TMAO (115-117). As with other gut-derived uremic toxins, poor TMAO clearance by dialysis is likely related to the broad tissue distribution of many of these compounds. As demonstrated in a recent post-hoc analysis from the HEMO study (116), increasing dialysis intensity in ESRD patients has little impact on chronic TMAO exposure in this population (117).

Given that dietary interventions and aggressive dialysis are unlikely to substantially lower TMAO concentrations in CKD patients, alternative strategies are currently being explored. A recently published investigation suggested that changes in the intestinal microbiota in CKD patients results in an increased production of TMAO

(118). Thus, one reasonable strategy to lower TMAO in CKD patients could be to promote a shift of the “pathogenic” bacterial flora to more symbiotic species with less capacity for TMA generation. Unfortunately, existing studies investigating the utility of prebiotics or probiotics for supporting the growth of more symbiotic bacterial flora in CKD patients have largely yielded disappointing results (119). A more targeted approach, inhibiting the specific bacterial enzymes that produce TMA, may be a more reasonable strategy. Such targeted therapies are currently undergoing testing in animals (120). Lastly, the results of our current study would support a need for further examination of therapies targeting hepatic FMO activity as an added approach for lowering TMAO concentrations in this population.

As with any study, the current investigation has both important strengths and limitations. Important strengths of our investigation include: use of a precise and extensively validated method for TMAO quantification, the inclusion of studies to assess hepatic FMO enzyme function, consideration of the impact of gender on the outcomes of interest, performing metabolic studies which included timed urine collections for the assessment of urinary TMAO excretion, and an overall study design that allows for the exclusion of potential confounders that are commonly encountered in human studies (i.e. dietary influences, genetic variability, and the presence of disease comorbidities). Important limitations of our work include: possible differences between human and mouse biology, lack of investigation into specific mechanisms driving the observed alterations in hepatic generation or renal clearance of TMAO, no assessment of FMO3 and FMO1 protein expression, and no assessment of how alterations to the intestinal microbiome may have altered TMA formation rates in CKD mice.

In summary, our observations in CKD mice suggest that both a decrease in renal clearance of TMAO and an increase in hepatic production of TMAO due to enhanced FMO3 activity may equally contribute to TMAO accumulation in CKD. If future studies confirm our findings in humans, it will be important to further explore the significance of enhanced TMAO formation in CKD and better understand how blockade of FMO3 activity may alter clinical outcomes in this setting.

CHAPTER 3: Intestinal Alkaline Phosphatase (IAP) and Tissue Non-Specific Alkaline Phosphatase (TNAP) Activity and Expression in Mouse Models of Chronic Kidney Disease

INTRODUCTION

In patients with CKD, systemic inflammation contributes to the presence of numerous comorbid conditions, including CVD, malnutrition, anemia, and mineral-bone defects (121). As described in Chapter 1, intestinal barrier defects resulting in the translocation of bacteria into the systemic circulation are hypothesized to contribute to the pro-inflammatory phenotype observed in CKD patients. Accumulating evidence supports a role of alkaline phosphatases in regulating the relative abundance of intestinal bacteria and maintaining intestinal barrier function to limit the activation of pro-inflammatory pathways; however, the contribution of these alkaline phosphatases to gut barrier function and inflammation in CKD has yet to be examined.

Alkaline phosphatases (ALP) are a family of GPI-linked metalloenzymes with phosphatase activity occurring in the extracellular space, and optimal protein activity occurring in alkaline environments. Despite a historical lack of understanding of the physiological role of ALP, physicians and scientists have been measuring ALP enzyme activity since the 1970s, when clinical studies first reported altered activity levels in patients with hepatobiliary disease and skeletal defects (122).

The isoforms of ALP expressed in the mouse include tissue non-specific alkaline phosphatase (TNAP), intestinal alkaline phosphatase (IAP), and embryonic alkaline phosphatase (Table 3.1). TNAP (*Akp2*) is expressed in the liver, kidney, and bone. In the liver, TNAP expression and activity is restricted to portal arteries and bile canaliculi

(123). Two ALP isoforms expressed in the intestine include *Akp3,* which is restricted to the duodenum, and *Akp6*, which is expressed globally throughout the small intestine. The highest expression of placental-like alkaline phosphatase (*Akp5*) occurs in the placenta, but has also been found in the serum (124), and ectopic expression is used as a marker of cancer in the reproductive organs (125,126). Chemical inhibitors are often employed to discern differences in isoform activity. For example, levamisole is used as an inhibitor of TNAP activity and L-phenylalanine is a potent inhibitor of both IAP (*Akp3*) and placental-like alkaline phosphatase (*Akp5*) activity (127).

Table 3.1 Mouse Alkaline Phosphatase Isoforms.

Protein Name	Gene Name	Chemical Inhibitors
Tissue Non-Specific Alkaline Phosphatase (TNAP)	*Akp2* (Liver/Kidney/Bone)	Levamisole
Intestinal Alkaline Phosphatase (IAP)	*Akp3* (Duodenum) and *Akp6* (Small Intestine)	L-phenylalanine
Placental-like Alkaline Phosphatase	*Akp5* (placenta)	L-phenylalanine

The role of TNAP in regulating bone and soft tissue calcification through the dephosphorylation of pyrophosphate has been established for decades (128); however, until recently, the natural substrates for ALP isoforms expressed in the kidney, intestine and liver have remained unknown. Due to its anionic nature, researchers speculated the bacterial endotoxin LPS may be an endogenous substrate of ALP. The negative charge of LPS would sequester free protons increasing the local pH allowing for optimal ALP activity, while the phosphates on the sugar residues of the lipid A motif are essential for LPS to bind to its receptor and trigger an inflammatory cascade can be removed by ALP (129). To investigate the role of ALP in detoxifying LPS, Polestra *et. al.* injected mice with sub-lethal doses of *E. coli* with and without levamisole, a non-specific inhibitor of ALP activity. Mice injected with *E. coli* and levamisole had a significant reduction in survival compared to mice injected with *E. coli* alone. In addition to this survival study, the authors utilized histochemistry techniques to elucidate that the duodenum and liver were capable of dephosphorylating LPS at a neutral pH, whereas using a phosphate substrate such as β-glycerophosphate, which lacks negative charge, required the reaction to occur at a basic pH between 9-10 (129).

Subsequent studies validated that IAP expression specifically was capable of enhancing the survival of rodents injected IP with *E. coli* or zebrafish exposed to LPS in tank water (130,131). In addition to LPS, IAP is capable of dephosphorylating nucleotide triphosphates ATP and UTP, bacterial DNA, and modifying the structure of flagella to limit the ability of these metabolites to stimulate an inflammatory response (132).

Deletion of *Akp3* in mice resulted in no gross abnormalities of the duodenum and ileum (133). Upon further investigation, *Akp3*$^{-/-}$ mice do display signs of low-grade

inflammation, including increased hepatic MHC II staining (134), increased serum endotoxin, and increased serum TNFα (135). It is plausible that this inflammatory phenotype is driven by subtle changes to the intestinal barrier, as upon oral-LPS challenge, more LPS translocates into the systemic circulation of the *Akp3*$^{-/-}$ mice compared to their wild-type counterparts (135). Lastly, there is a shift in the relative abundance of the resident bacterial phyla in the stool collected from *Akp3*$^{-/-}$ mice (136,137), possibly due to a local accumulation of ATP that results in limited bacterial growth (138). These data suggest a role of IAP in fortifying the intestinal barrier and maintaining the relative abundance of intestinal microbe populations; however, studies dissecting the mechanism for these proposed roles have yet to be conducted.

The activity of IAP is decreased in an array of diseases, including those with intense intestinal inflammation, such as inflammatory bowel disease (139), celiac disease (140) and cystic fibrosis (141), to chronic diseases exhibiting more subtle defects in intestinal barrier function, such as diabetes (142) and alcoholic-liver disease (143). Moreover, oral administration of IAP to animal models of metabolic endotoxemia (135), alcohol-induced liver injury (144), and cystic fibrosis (141), appears to limit inflammation and attenuate disease progression.

Based on the above observations suggesting a critical function of IAP in the preservation of gut barrier function and microbiota composition, we hypothesized that CKD would be accompanied by decreased IAP activity, resulting in impaired intestinal barrier function in this setting. Furthermore, we speculated that IAP therapy would attenuate the inflammatory phenotype observed in CKD. Outside of researchers studying the role of ALP in bone and soft tissue calcification, the tissue expression

patterns and activity of various ALP isoforms have yet to be explored in CKD models. The primary purpose of the studies outlined in this chapter was to characterize the expression patterns of ALP isoforms in: (1) the duodenum (due to the high expression of IAP at this site), (2) in stool collected from the ileum (to determine whether IAP protein may be secreted into the intestinal lumen), and (3) the liver (a major site of LPS detoxification).

METHODS

Animal Model

We utilized two CKD mouse models throughout this study. First, readouts described below were explored in the collagen 4α3 (*Col4α3*) knockout model on the SVJ/129 background. The *Col4α3*$^{-/-}$ mice are a model of the human Alport disease, while *Col4α3*$^{+/+}$ littermates served as non-CKD controls. We validated results observed in the *Col4α3* model with an adenine-induced model of CKD. Briefly, C57BL6/J mice starting at 6 weeks of age were fed a diet containing 0.2% adenine for 12 weeks, and mice maintained on an identical diet lacking adenine supplementation served as non-CKD controls. Non-CKD and CKD mice were housed in separate cages throughout the duration of these studies. At the time of sacrifice, tissue was either fixed in 4% PFA or snap frozen and stored at -80°C for further analysis.

Quantitative PCR

Total RNA was isolated from tissue homogenized with Tri-Reagent (Molecular Research Center, Cincinnati, OH) following manufacturer's protocol. First-strand cDNA was synthetized from 1 μg of RNA using iScript (Biorad, Hercules, CA). Quantitative

PCR was performed using 1X iQ™ SYBR® Green Supermix (Biorad, Hercules, CA), contained 40 ng cDNA, and 150 nM of each primer in 20 μl final volume. Quantification was calculated using the Pfaffl method (104), and the threshold cycle (Ct) of each gene product was normalized to the Ct for *HPRT*. Primer sequences used were: *LBP* F-CCACAGATGGAGATCGAAGG and R-TGAGGCAAATACATTAGTGACCA; *CD14* F-AAAGAAACTGAAGCCTTTCTCG and R-AGCAACAAGCCAAGCACAC; *IL1β* F-GCCACCTTTTGACAGTGATGAG and R-GACAGCCCAGGTCAAAGGTT; *TLR4* F-CGCTTTCACCTCTGCCTTCACTACAG and R-ACACTACCACAATAACCTTCCGGG TC; *Akp3* F-CATGGACCGCTTCCCATA and R-CTTGCACTGTCTGGAACCTG; *Akp6* F-GGACCCAGCAGTAACTCACC and R-TGGTCAATACGACCCCCTTC; *TNAP* F-CCTGACTGACCCTTCGCTCT and R-CTGCTTGGCCTTACCCTCAT; *HPRT* F-TGATAGATCCATTCCTATGACTGTAGA and R-AAGACATTCTTTCCAGTTAAAG TTGAG.

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was measured by fluorometric assay using 4-methylumbelliferyl phosphate disodium salt as a phosphate substrate (Abcam, Cambridge, UK). Following manufacturer's protocol, tissue was homogenized and diluted using provided lysis buffer. To discern IAP activity from other possible ALP sources, samples were run in parallel with and without 10 mM L-phenylalanine, an inhibitor of IAP activity. The L-phenylalanine sensitive IAP activity was calculated by subtracting the activity value obtained in wells with L-phenylalanine from those without L-phenylalanine. In intestinal tissue, this L-phenylalanine sensitive activity will be labeled IAP activity whereas in the liver, bone and serum, this activity will be labeled as

L-phenylalanine sensitive ALP activity, since IAP is not expressed by these sources. Protein activity was normalized to the total protein content of the sample, determined by BCA assay (Pierce, Rockford, IL).

Immunoblotting

Tissue used for immunoblotting was previously lysed for the ALP activity assay described above. Between 30 and 100 μg of protein was diluted in NuPAGE® reducing buffer (Life Technologies, Carlsbad, CA), incubated at 70°C for 10 minutes, and resolved on 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA). Proteins were transferred onto PVDF membrane using semi-dry transfer. Membranes were stained with Ponceau S, blocked in 5% milk, and then incubated with either primary IAP (Abcam, Cambridge, UK; ab97532) or TNAP (Abcam, Cambridge, UK; ab108337) antibodies overnight. Blots were incubated with HRP-conjugated secondary antibodies and Enhanced Chemiluminescent substrate was used to generate luminescence (GE Healthcare, Piscataway, NJ). Blots were visualized using GE Amersham Imager 600 (GE Healthcare, Piscataway, NJ), and densitometry analysis was performed using Image J software (National Institute of Health, USA). Protein quantification was normalized to total protein content after Ponceau S staining of the membrane.

IAP Immunohistochemistry

Tissues were fixed in 4% PFA for 24 hours, paraffin embedded, then cut into 5 μm sections. After deparaffinization, heat-mediated antigen retrieval using citrate buffer (Sigma, St. Louis, MO) at pH 6.0 was performed. Endogenous peroxidase activity was quenched with 3% H_20_2, then tissue sections were blocked and incubated with the IAP primary antibody overnight at 4°C. Sections were washed and incubated with HRP-

conjugated secondary antibody (ImmPRESS, Vector Laboratories, Burlingame, CA) and visualized using Diaminobenzidine (ImmPACT, Vector Laboratories, Burlingame, CA).

Statistical Analysis

Comparisons between non-CKD and CKD animals were evaluated by Student's t-test, or Mann-Whitney test for data exhibiting a non-normal distribution. Calculations were performed using Prism 7 (GraphPad Software, San Diago, CA) and presented as mean $\pm$ SD.

RESULTS

Inflammatory phenotype of the liver and spleen in CKD mice

To first assess a possible inflammatory phenotype originating from intestinal barrier dysfunction, we performed gene expression analysis of LPS signaling proteins (LBP, CD14, and TLR4) and the inflammatory cytokine IL1β. Luminal content of the digestive tract is absorbed into systemic circulation either through the portal vein or the lymphatic system; therefore, we performed qPCR analysis on the livers and spleens collected from non-CKD (*Col4*α*3*$^{+/+}$) and CKD (*Col4*α*3*$^{-/-}$) mice. Kidney disease resulted in a 3.5-fold increase in liver gene expression of LBP (Figure 3.1A, $P<0.05$), a 2-fold increase in CD14 (Figure 3.1B, $P<0.05$), and a trend toward increased IL1β gene expression (Figure 3.1 C; $P=0.09$). Within the spleen, we observed a significant increase in IL1β (Figure 3.1 D; $P<0.05$) and TLR4 gene expression (Figure 3.1 E; $P<0.05$).

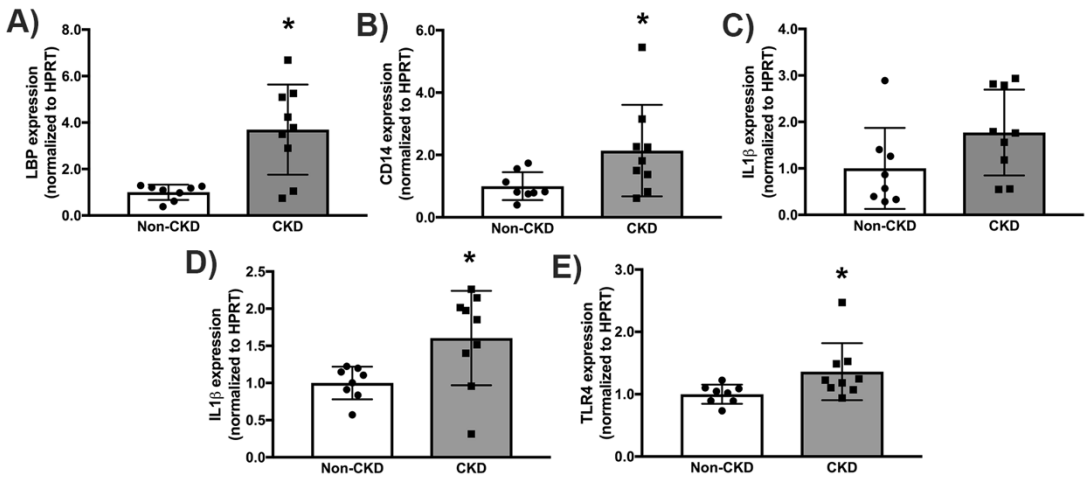

Figure 3.1 Quantitative-PCR analyses of inflammatory markers in the (A-C) liver and (D and E) spleen of *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) mice. Kidney disease resulted in a significant increase in liver **(A)** *LBP* and **(B)** *CD14* gene expression. A slight elevation in **(C)** *IL1β* was observed in the livers of CKD mice. Additionally, there was a significant increase in the gene expression of **(D)** *IL1β* and **(E)** *TLR4* in the spleens of CKD mice. *P<0.05 vs non-CKD controls

Small intestine IAP activity and expression in CKD mice

In the *Col4α3* model, there was a 50% reduction in duodenum IAP activity in CKD mice compared to non-CKD controls (Figure 3.2 A; CKD 311,353 mU/mg protein vs non-CKD 610,074 mU/mg protein, $P<0.05$). In the adenine-induced model of CKD, a substantial reduction of duodenum IAP activity was also observed (Figure 3.2 B; CKD 208,709 mU/mg protein vs non-CDK 559,826 mU/mg protein, $P=0.11$). Further analysis of duodenum collected from $Col4\alpha3^{+/+}$ and $Col4\alpha3^{-/-}$ revealed no change in the gene expression of IAP isoforms expressed in the duodenum, *Akp3* and *Akp6* (Figure 3.2 C and D). To determine the reactivity of our IAP antibody, we immunoblotted duodenum tissue of $Akp3^{+/+}$ and $Akp3^{-/-}$ mice and no staining occurred in the $Akp3^{-/-}$ mice. No change in duodenum IAP protein expression occurred with the induction of CKD (Figure 3.2 G and F). Furthermore, immunohistochemistry for IAP in non-CKD mice revealed diffuse cytoplasmic staining that was lost in the duodenum of CKD mice (Figure 3.3 A). Under low magnification of the duodenum, IAP protein staining appeared to be shed and possibly retained within the mucus layer of CKD mice (Figure 3.3 B).

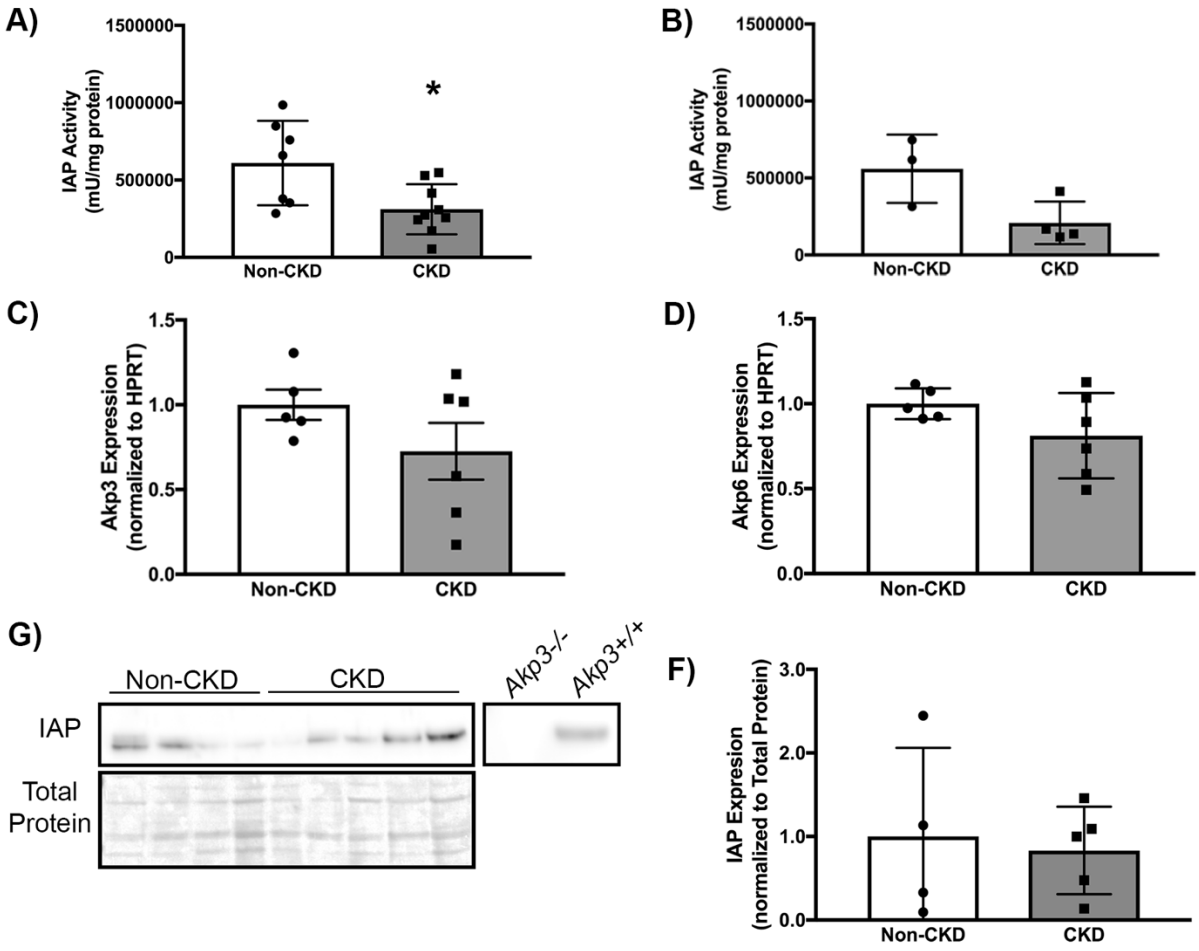

Figure 3.2 Duodenum IAP activity, gene and protein analysis. (A) Within the duodenum of *Col4α3*$^{-/-}$ (CKD) mice, there was a significant decrease in IAP protein activity compared to *Col4α3*$^{+/+}$ mice (non-CKD). **(B)** This same trend was observed in mice maintained on a diet containing adenine for 12 weeks to induce kidney injury (P=0.11). There were no changes in gene expression of the duodenum IAP isoforms, **(C)** *Akp3* and **(D)** *Akp6*. **(G, F)** Immunoblot analysis and quantification for IAP protein expression proved there to be no difference in whole-tissue lysate protein expression of IAP within the duodenum of *Col4α3*$^{-/-}$ mice. *P<0.05 vs non-CKD controls

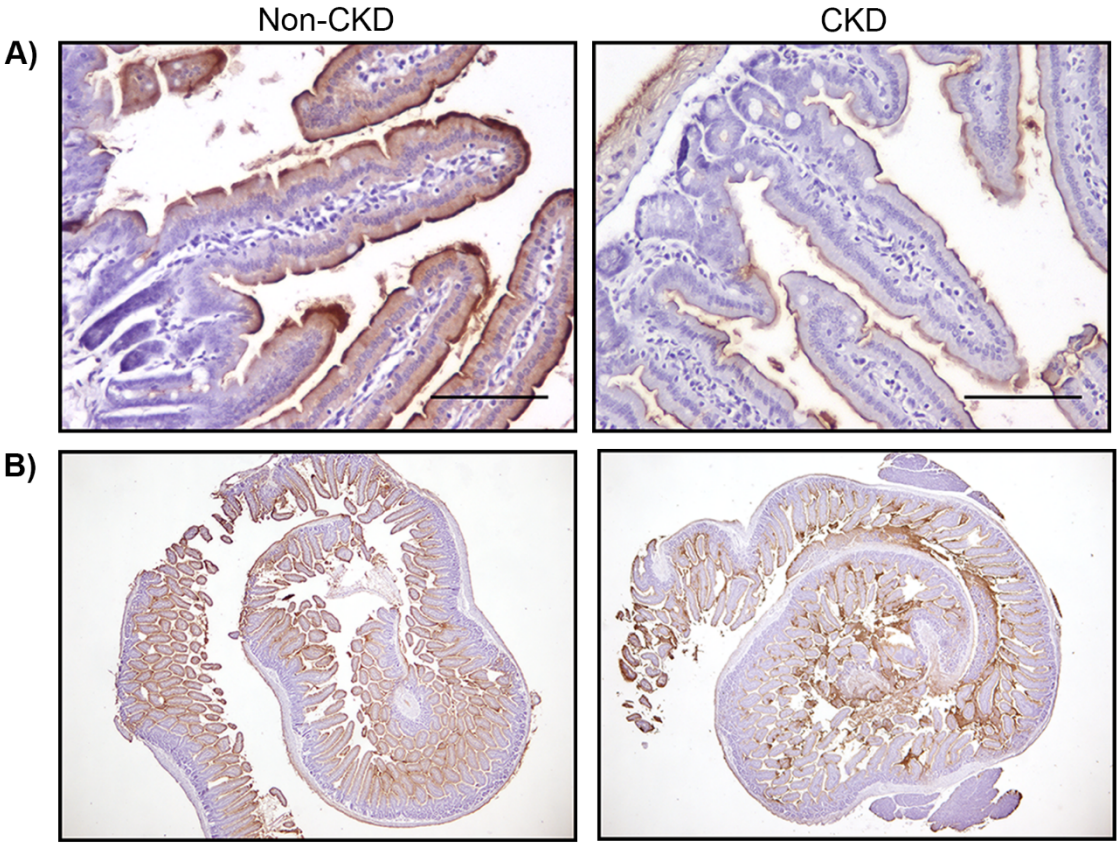

Figure 3.3 Cellular localization of duodenum IAP in *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) mice. (A) In the duodenum of non-CKD mice, IAP protein expression is predominantly localized to the apical membrane, with diffuse cytoplasmic staining. CKD mice, while still having IAP staining at the apical membrane, was void of cytoplasmic staining. (scale bar=100 μM, 200X magnification). **(B)** Low power images demonstrated IAP staining in the intestinal lumen of CKD mice (20X magnification).

To explore the possibility of IAP protein being secreted from the duodenum, and retained within the stool, we examined IAP activity and protein expression of luminal content collected from a downstream intestinal segment, the ileum. We observed an approximate 2-fold increase in both IAP activity (Figure 3.4 A; CKD 9,371,407 mU/mg protein vs non-CKD 4,983,876 mU/mg protein, P=0.07) and protein expression (Figure 3.4 B and C; P=0.4). Additionally, there was an approximate 2-fold increase in IAP activity in ileum tissue cleaned of stool (Figure 3.5; CKD 164,535 mU/mg protein vs non-CKD 90,809 mU/mg protein, P=0.28).

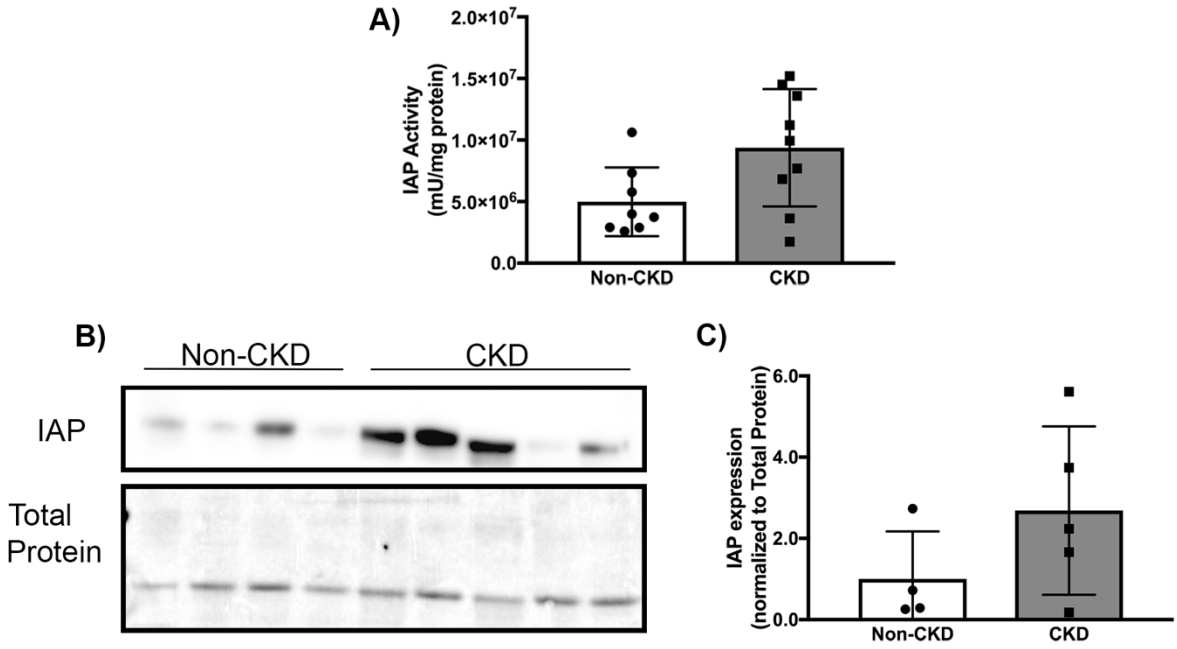

Figure 3.4 IAP activity within stool collected from the ileum of *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) mice. (A) In CKD mice, increased IAP activity in stool obtained from the ileum of mice (P=0.07). **(B, C)** The rise in activity was accompanied by an increase in protein expression of IAP within the stool, by immunoblot analysis.

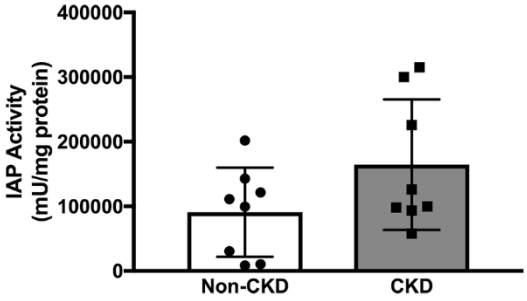

Figure 3.5 Ileum tissue activity in *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) mice.

There was a modest increase in IAP activity in ileums collected from CKD compared to non-CKD mice P=0.28.

Hepatic ALP activity and expression in CKD mice

In the *Col4α3* model, there was a significant increase in hepatic TNAP (Figure 3.6 A; CKD 3,140 mU/mg protein vs non-CKD 294.1 mU/mg protein, P<0.001) and L-phenylalanine sensitive ALP activity in CKD mice (Figure 3.5 B; CKD 260.3 mU/mg protein vs non-CKD 54.7 mU/mg protein, P<0.001). These results were corroborated in the adenine-induced CKD mouse model for hepatic TNAP (Figure 3.6 C; CKD 18,017 mU/mg protein vs non-CKD 1,622 mU/mg protein; P<0.01) and L-phenylalanine sensitive ALP (Figure 3.6 D; CKD 2,418 mU/mg protein vs non-CKD 129.5 mU/mg protein; P<0.05) activity. Kidney injury resulted in no change in liver TNAP gene expression in *Col4α3*$^{-/-}$ mice (Figure 3.7 A), however, there was a 50% increase in TNAP protein expression (Figure 3.7 B and C, P<0.05). Immunoblotting liver lysate with the IAP antibody revealed two bands at a molecular weight near 150 kDa that was also observed in liver tissue from *Akp3*$^{-/-}$ mice (Figure 3.8 A), possibly suggesting detection of an alternate ALP isoform. Additionally, there was an increase in liver staining with the IAP antibody in both the *Col4α3* model (Figure 3.8 B) and the adenine-induced model of CKD (Figure 3.8 C).

Systemic ALP Activity

To determine whether the changes to ALP activity were occurring systematically or restricted to the gut-liver axis of CKD mice, we measured ALP activity in the bone and serum of CKD animals. No change in TNAP and L-phenylalanine sensitive ALP activity was observed in the bone (Figure 3.9 A and B) or serum (Figure 3.9 C and D) of CKD animals compared to non-CKD controls.

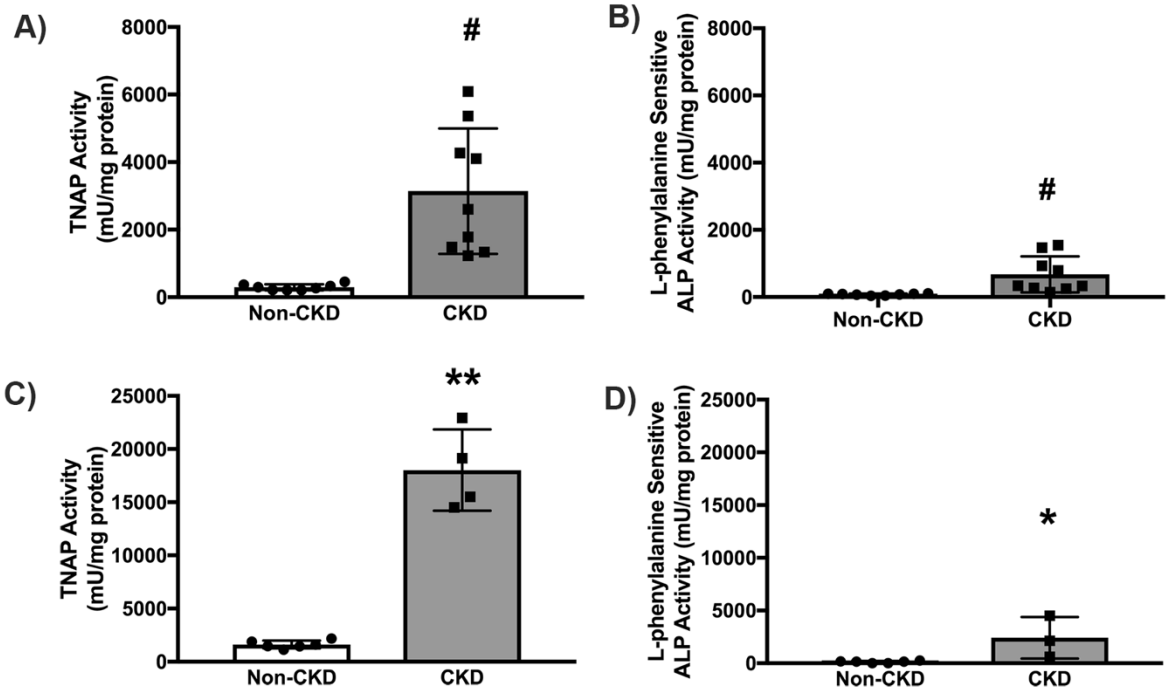

Figure 3.6 Liver ALP activity. In the *Col4α3*$^{-/-}$ (CKD) mice, there is a significant increase in liver **(A)** TNAP and **(B)** L-phenylalanine sensitive ALP activity compared to *Col4α3*$^{+/+}$ (non-CKD) mice. Furthermore, in an adenine-induced CKD model, this finding of increased liver **(C)** TNAP and **(D)** L-phenylalanine sensitive ALP activity is replicated. *P<0.05, **P<0.01, #P<0.001 vs non-CKD controls.

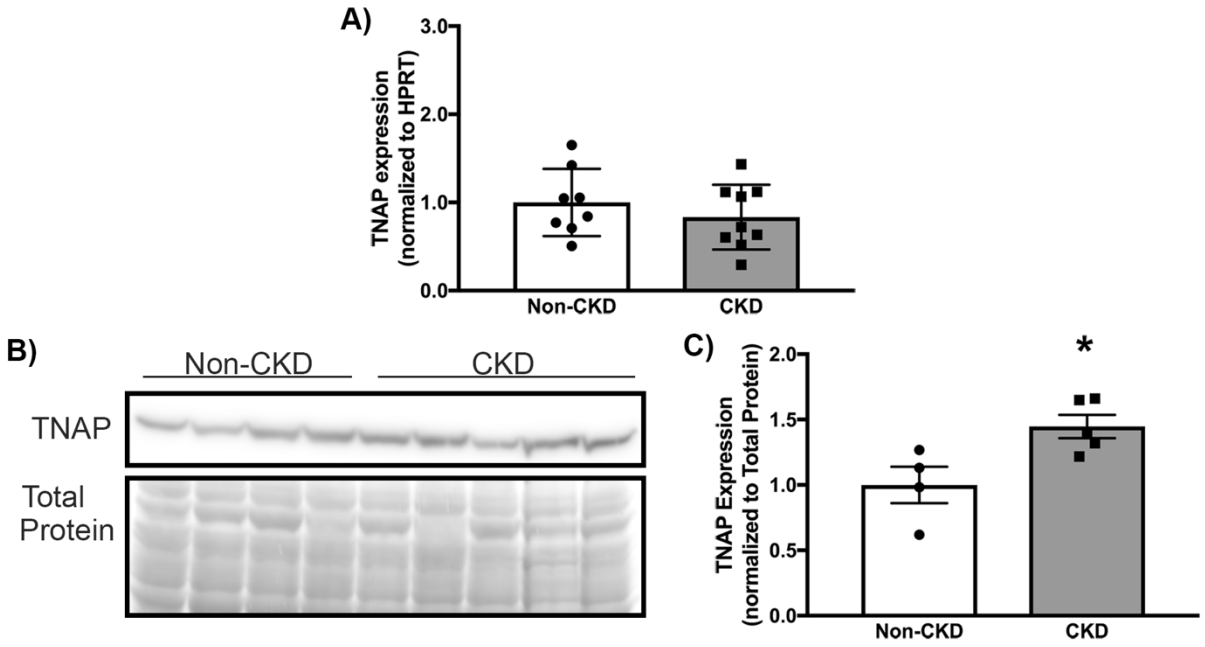

Figure 3.7 Hepatic TNAP gene and protein expression in *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) mice. (A) Hepatic TNAP gene expression was unchanged in the livers of CKD mice compared to non-CKD mice. **(B, C)** Immunoblot analysis of livers revealed a slight and significant increase in TNAP protein expression in CKD mice compared to non-CKD mice. *P<0.05 vs non-CKD controls.

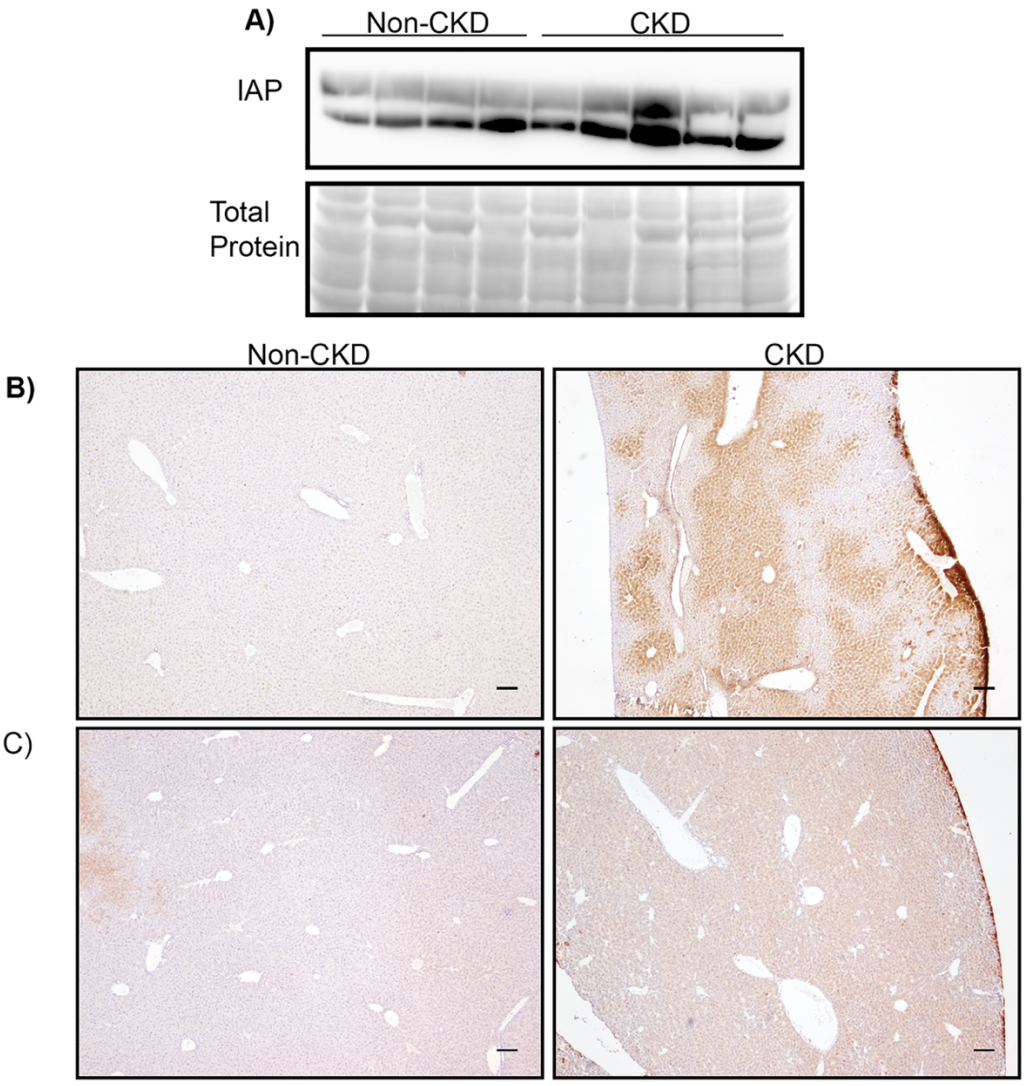

Figure 3.8 Protein analysis of livers with IAP antibody. (A) Immunoblot analysis of livers from *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) for IAP protein expression. Two bands appeared at a molecular weight of 150 kDa. These same bands appeared in liver tissue collected from the *Akp3*$^{-/-}$. Through immunohistochemistry staining of the liver with the IAP antibody, the **(B)** *Col4α3* model of CKD and **(C)** adenine-induced model of CKD had increased staining compared to non-CKD controls (scale bar = 100 μM, 40X magnification).

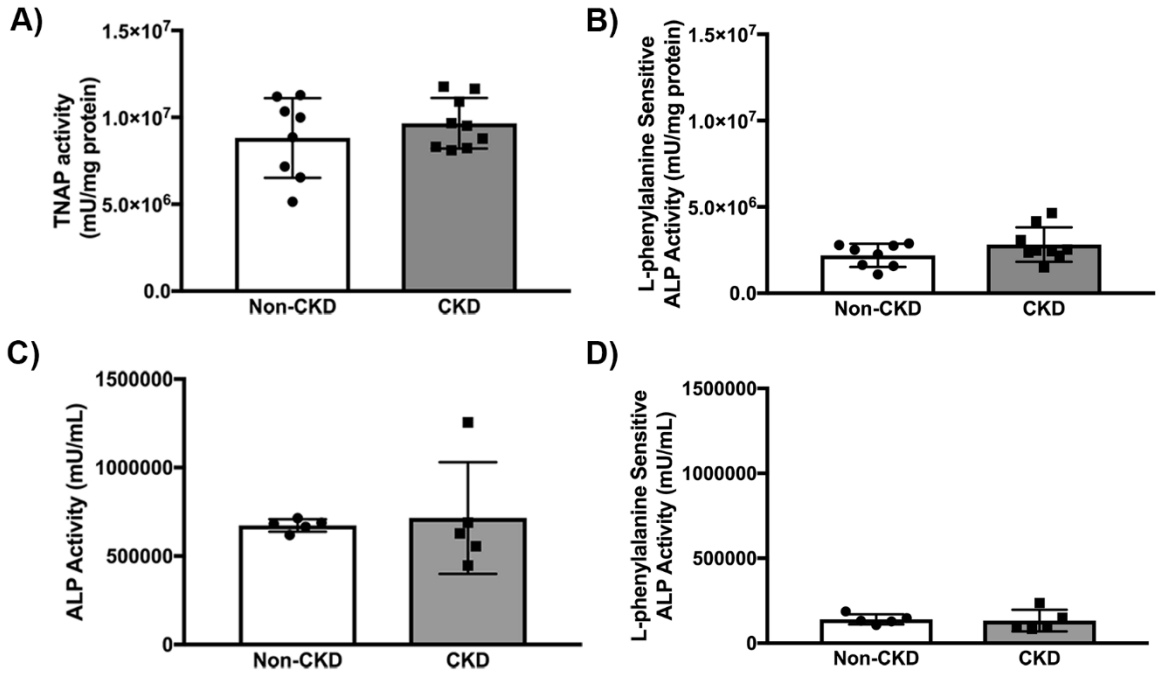

Figure 3.9 TNAP and IAP activity in bone and circulation of *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) mice. Within the calvaria, there was no difference in **(A)** TNAP and **(B)** L-phenylalanine sensitive ALP activity. Likewise, there was no observed difference in serum **(C)** ALP and **(D)** L-phenylalanine sensitive ALP activity.

DISCUSSION

IAP and TNAP activity are implicated in maintaining intestinal barrier function and limiting systemic inflammation. Moreover, decreased IAP activity is observed in disease states characterized by a pro-inflammatory phenotype and IAP therapy attenuates systemic inflammation. CKD patients exhibit evidence of intestinal barrier dysfunction that likely contributes to a pro-inflammatory phenotype; however, the role of IAP in CKD-related barrier dysfunction has yet to be explored. The current study was designed to characterize the expression and activity of IAP in the intestine, and hepatic ALP expression and activity, as the liver is a major site of detoxification of LPS and other bacterial byproducts.

Our first notable observation in this study is a decrease in duodenum IAP activity in CKD (*Col4α3*$^{-/-}$) mice compared to non-CKD controls (*Col4α3*$^{+/+}$) (Figure 3.2 A); this trend of decreased duodenum IAP activity was confirmed in an alternative CKD model (adenine diet) (Figure 3.2 B). Furthermore, decreased IAP activity in CKD mice is not due to decreased gene or protein expression, instead suggesting altered post-translation modification of the IAP protein, altered cellular localization, or the presence of a factor capable of modifying the enzymatic activity of IAP. Interestingly, unlike TNAP activity, IAP activity is not dependent on its glycosylation state (145); therefore, our finding of a loss of cytoplasmic expression within duodenal enterocytes of CKD mice would suggest this loss of IAP activity is accompanied by a major shift in the cellular localization of IAP expression. (Figure 3.3). A possible mechanism responsible for this phenomenon could be altered expression of bacterial phospholipases capable of cleaving the GPI-anchor of the IAP protein. In turn, cleaved protein may get trapped in

the mucus layer of the duodenum and lose its enzymatic activity, or maintain its activity and travel downstream into stool from distal intestinal segments, such as the ileum (Figure 3.4). An alternative mechanism resulting in increased activity and protein expression we observed within the stool is the production of IAP by the ileum. In support of this scenario, we do observe a modest increase in IAP activity in CKD mouse ileum that has been washed of fecal contents (Figure 3.5).

Most convincingly, we have shown that both TNAP and an ALP isoform sensitive to L-phenylalanine inhibition are increased in the livers of *Col4α3* and adenine-induced CKD mouse models (Figure 3.5). Increased activity was accompanied by increased whole-tissue protein levels of TNAP, and increased IHC staining with an IAP antibody that may be due to increased placental-like ALP expression. A similar phenotype of increased hepatic ALP activity is observed in other inflammatory diseases including hepatic diseases (146,147), cystic fibrosis (148), and *E. coli* induced bacteremia in humans (149).

Interestingly, the significant increase in hepatic ALP activity in CKD mice was not reflected in overall serum phosphatase activity. This may be due to liver ALP only being a minor source of circulating phosphatase activity in mice (123). Consistently, CKD patients do not regularly exhibit increased serum ALP activity, except in the setting of concomitant high bone turnover states (150). An alternative explanation for this observation may be a lack of liver ALP solubility upon kidney injury. An analogous scenario was described in rats undergoing hepatectomy compared to rats undergoing bile duct ligation. In both scenarios, increased liver ALP activity at the tissue level was observed, but a change in circulating ALP activity was only present in the animals

undergoing bile duct ligation. The authors of this investigation went on to speculate a role of bile acids in solubilizing liver ALP for its release into the bloodstream (151).

While we provide data that IAP and TNAP activity is altered in the gut-liver axis of CKD mice, we are unsure of the clinical significance of these finding and mechanisms regulating intestinal and liver ALP activity. To address these gaps in our knowledge, we have first designed studies examining the impact of kidney injury in the *Akp3*$^{-/-}$ mice, to determine whether the loss of Akp3 exacerbates the pro-inflammatory phenotype observed in CKD mice. Additionally, we have designed experiments to determine whether IAP therapy can reduce inflammation in CKD mice. In separate studies, we plan to determine the significance of increased hepatic ALP activity in CKD mice by determining the effect of inhibition of enzyme activity on pro-inflammatory pathways and endotoxemia. ALP inhibition will be accomplished using a novel TNAP inhibitor, SBI-425 (152). We hypothesize that the upregulation of liver ALP in CKD is a compensatory mechanism to limit inflammation driven by increased entry of bacterial endotoxins into the portal circulation when gut barrier function is compromised; therefore, we anticipate that inhibition of ALP activity in the setting of CKD would intensify the pro-inflammatory phenotype. Regulation of ALP activity will be further explored in Chapter 4 of this dissertation.

This study has both strengths and weaknesses. Strengths of these studies include: the use of two separate CKD models to validate important observations, the use of *Akp3*$^{-/-}$ tissue to validate the specificity of our antibody, and ALP activity data being interpreted in conjunction with expression data. Weaknesses of this study include: not knowing what isoform our antibody is reacting with in the liver, the lack of TNAP IHC

staining within the liver, and overall ALP activity staining within the liver to determine if hepatic IHC staining localizes with the actual phosphatase activity.

In summary, our studies provide evidence that decreased IAP activity in CKD animals may be a contributing factor to altered intestinal biology observed after kidney injury. It is now important to explore the clinical significance of the changes in activity patterns, and whether IAP therapy can attenuate the inflammatory phenotype observed in CKD.

CHAPTER 4: Regulation of Alkaline Phosphatase Activity by Cecal Content from CKD mice

INTRODUCTION

Despite extensive research characterizing intestinal alkaline phosphatase (IAP) activity in disease states accompanied by intestinal barrier defects, studies exploring the mechanisms regulating IAP activity and expression are sparse. The majority of existing data does support some role of intestinal microbes in regulating IAP expression. For example, in both germ-free zebrafish and germ-free mice, IAP activity or gene expression is diminished (130,153). Furthermore, oral administration of the bacterial endotoxin, LPS, stimulates intestinal ALP activity within 2 hours of administration; however, this activity resolves within 6 hours (154) possibly due the production of inflammatory cytokines IL1β and TNFα, as these cytokines reduce IAP activity *in vitro* (155). Moreover, fasting animals demonstrate significantly lower duodenum IAP activity, with refeeding resulting in a rescue of this phenotype (156). Finally, unpublished observations from our lab suggest that co-housing CKD mice with non-CKD littermates results in a preservation of duodenal IAP activity in CKD animals. Taken together, these studies all suggest a potential role of the intestinal microbiota in regulating duodenum IAP activity.

Regulation of hepatic ALP activity is mostly studied in the context of liver disease, with little knowledge of ALP activity changes in other disease states. With liver injury, it is hypothesized that translocation of bacterial endotoxins from the gut lumen into portal circulation stimulates liver ALP activity. Furthermore, intraperitoneal LPS injection into wild-type mice results in increased hepatic TNAP gene expression (157);

however, ALP activity was not reported in these studies. Alternatively, bile acids are known to induce ALP activity in cultured rat hepatocytes (158), and biliary obstruction leads to a dramatic increase in hepatic ALP activity; yet, it is still unclear if bile acids are a direct *in vivo* regulator of hepatic ALP activity.

We hypothesized that alterations to the intestinal lumen contents (i.e. relative microbiota content or bile acid composition) in CKD result in decreased duodenum IAP activity, and a compensatory increase liver ALP enzymatic activity. To test this hypothesis, we performed fecal transplant studies to determine if stool from *Col4α3*$^{+/+}$ (non-CKD) and *Col4α3*$^{-/-}$ (CKD) mice could alter intestinal and hepatic ALP activity in wild-type recipients. Additionally, we injected wild-type mice with LPS to determine whether this bacterial byproduct alone can stimulate liver ALP activity.

METHODS

Fecal Transplant Study Design

Cecal content was collected from *Col4α3*$^{+/+}$ and *Col4α3*$^{-/-}$ mice at the time of sacrifice and snap frozen for later use. Cecal stool samples from three mice of the same genotype were pooled (i.e. 3 male *Col4a3*$^{+/+}$ were combined and 3 male *Col4a3*$^{-/-}$ were combined) and diluted 1:10 by weight in PBS + 50% glycerol to form the non-CKD and CKD fecal treatments. Immunoblot analyses of these samples were performed to determine whether any IAP protein was present in the supernatant.

As depicted in Figure 4.1, prior to fecal transplant, male C57BL/6J mice were administered an antibiotic cocktail (1 g/L ampicillin, 1 g/L neomycin, 1 g/L metronidazole, and 0.5 g/L vancomycin) through their drinking water for 2 weeks. At the end of this 2-week period, mice were randomized to receive either 150 μL of non-CKD

or CKD cecal content for a 3-week period. Prior to gavage, processed cecal stool samples were further diluted 1:5 in PBS. Oral gavage of fecal content was performed 3 times the first week (MWF), then once per week for the final 2 weeks (M). At the time of sacrifice, blood and tissue were collected and either fixed in 4% PFA or snap frozen and stored at -80°C.

Intraperitoneal LPS Injection

Wild-type C57Bl/6J mice were I.P. injected with 2 mg/kg of LPS (*E. coli*) or vehicle. Mice were sacrificed 6 hours after injection and tissue was snap-frozen and stored at -80°C for further analysis.

Tissue Analysis

All methods for kidney histology were described in detail in Chapter 2, whereas methods for qRT-PCR, alkaline phosphatase activity, immunoblotting, and immunohistochemistry were described in Chapter 3.

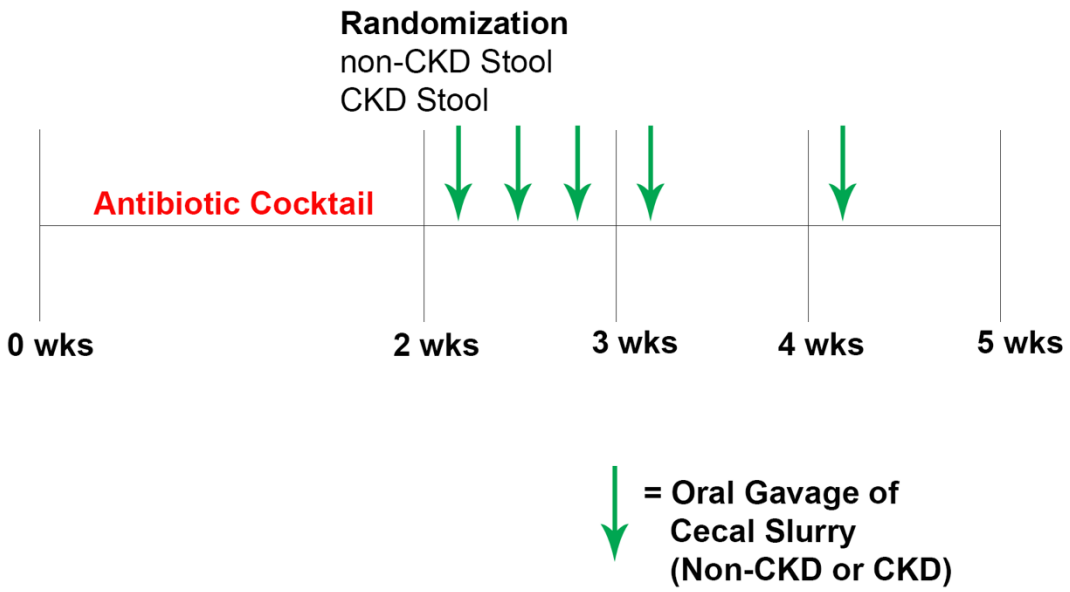

Figure 4.1 Fecal transplant study design. Male C57BL/6J mice at 10 weeks of age were administered an antibiotic cocktail containing 1 g/L ampicillin, 1 g/L neomycin, 1 g/L metronidazole, and 0.5 g/L vancomycin for 2 weeks. After 2 weeks, mice were randomized to receive processed cecal contents from previously sacrificed non-CKD or CKD mice. Mice were administered the fecal slurry by oral gavage three times during the first week of treatment, then once weekly for the final two weeks. After 5 weeks, the mice were sacrificed for blood and tissue collection.

RESULTS

IAP Expression in Cecal Content and Kidney Histology

There was no detectable IAP protein expression in the non-CKD and CKD fecal slurry (Figure 4.2 A). Additionally, there was no sign of kidney damage in the recipient mice (Figure 4.2 B).

IAP Protein Activity and Expression in the Small Intestine

Fecal transplant of CKD stool resulted in no change in IAP activity within the duodenum or stool content of the ileum compared to wild-type mice receiving non-CKD stool (Figure 4.3 A and B). There was a 7-fold increase in duodenum IAP protein expression in wild-type mice receiving CKD stool (Figure 4.3 C and D, $P<0.05$). IAP immunohistochemistry staining revealed that mice receiving non-CKD stool demonstrated strong staining at the apical membrane along with diffuse cytoplasmic staining of enterocytes, which is similar to the baseline staining patterns observed in non-CKD mice described in Chapter 3. In contrast, duodenal sections from the mice receiving CKD stool demonstrated apical membrane staining of enterocytes that was comparable to mice receiving non-CKD stool; however, cytoplasmic staining of enterocytes appeared more diffuse in mice treated with CKD stool (Figure 4.3 E).

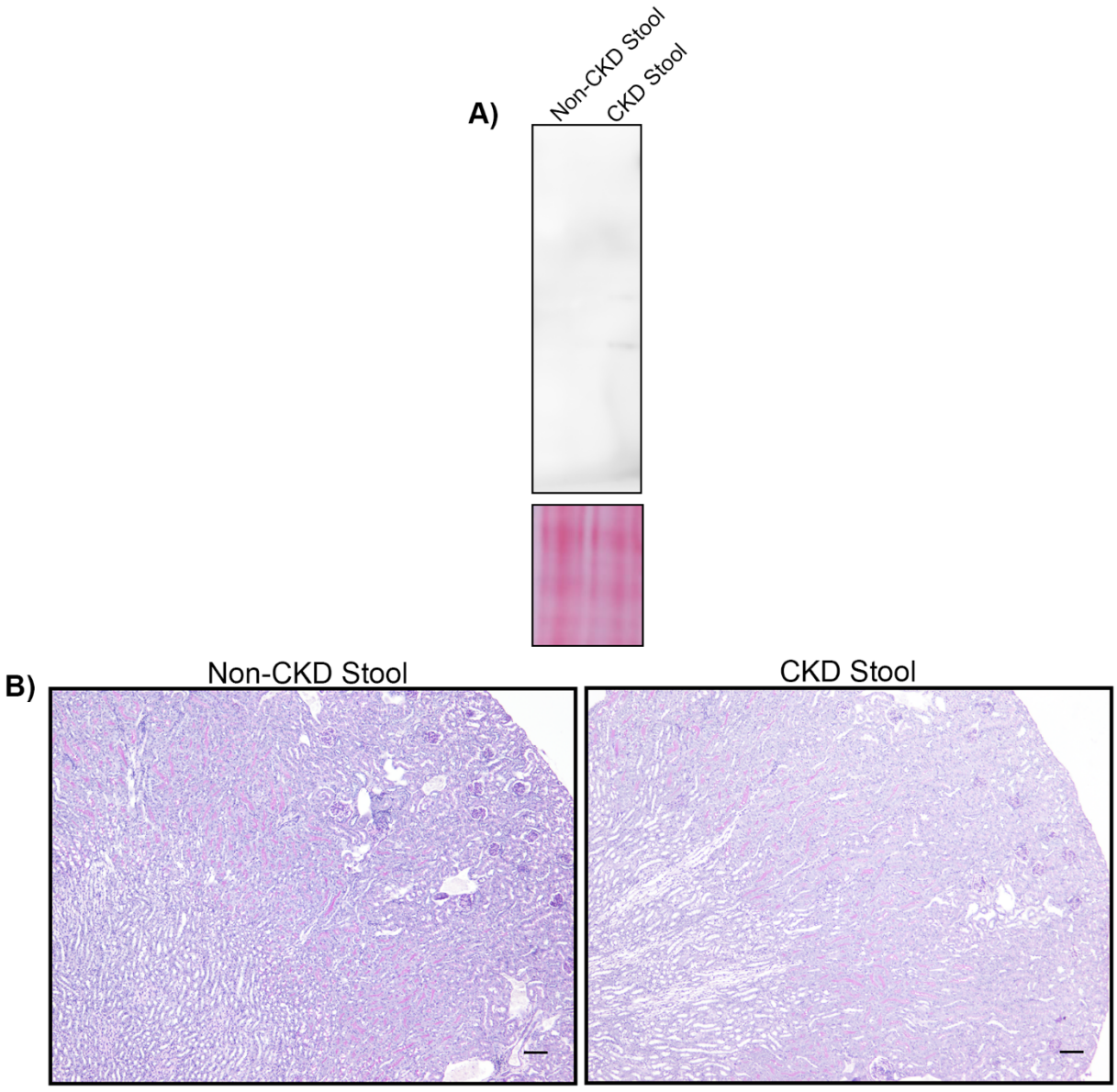

Figure 4.2 IAP expression in fecal content and kidney histology. (A) Immunoblot analysis revealed no detectable IAP expression in the cecal content pooled from non-CKD and CKD mice. **(B)** Fecal transplant did not result in overt kidney damage\ as observed by PAS staining (scale bar = 100μm, 40X magnification).

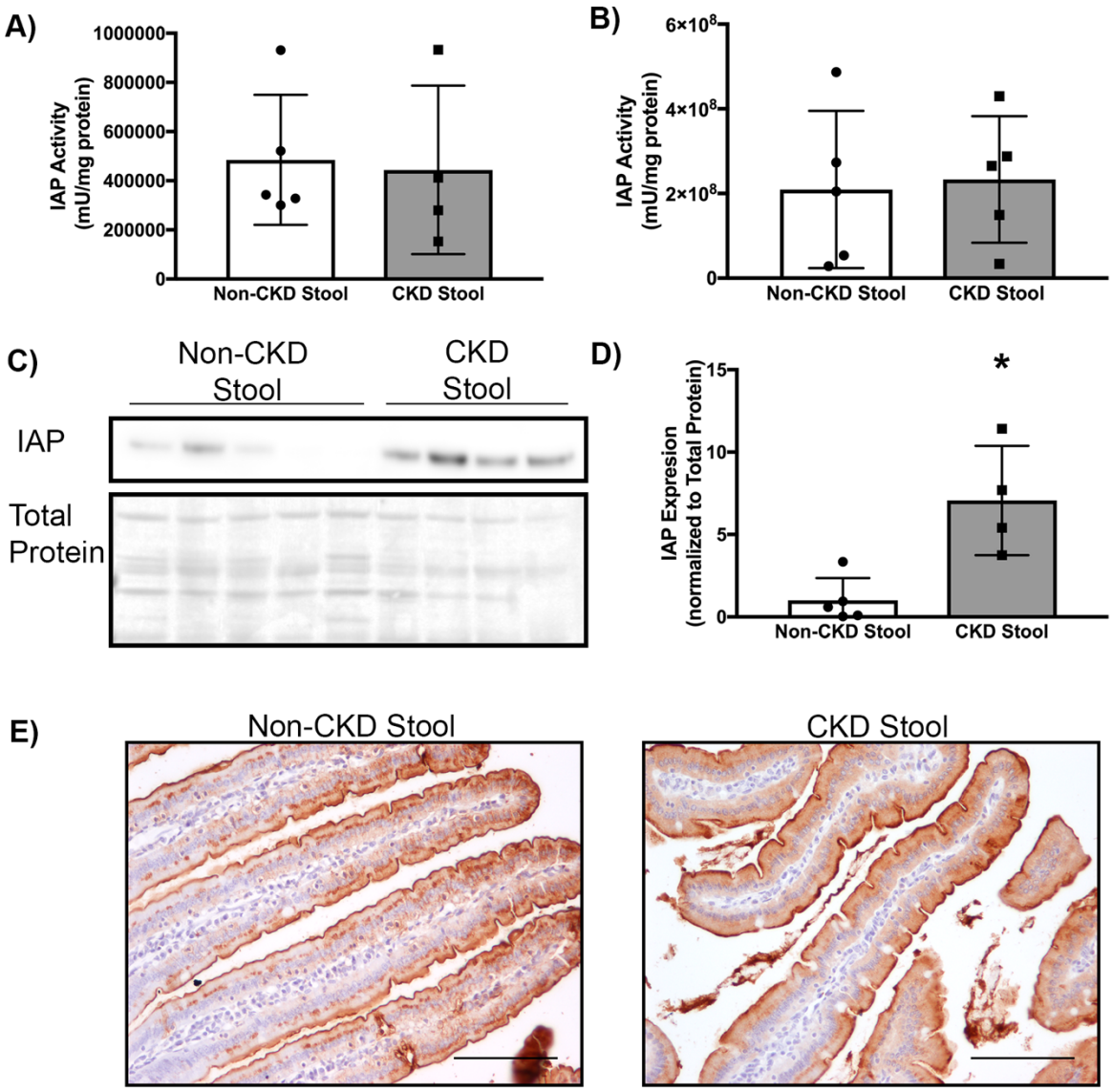

Figure 4.3 Intestinal IAP activity and expression. There was no difference in IAP activity in the **(A)** duodenum or **(B)** stool collected from the ileum of wild-type mice receiving CKD stool compared to those that received non-CKD stool. **(C, D)** There was a significant increase in IAP protein expression in mice receiving CKD stool. **(E)** Immunohistochemistry staining for IAP revealed more diffuse cytoplasmic staining in the mice receiving CKD stool versus those receiving non-CKD stool (scale bar = 100 μm, 200X magnification). *P<0.05 vs non-CKD stool controls

Liver ALP Activity and Expression

Fecal transplant of CKD stool into wild-type mice did not elicit an inflammatory phenotype in the liver, as we previously observed in our CKD animals (Chapter 3). Briefly, comparing wild-type mice receiving CKD stool versus non-CKD stool, there was no difference in hepatic gene expression for *LBP*, *CD14*, *TLR4*, or *IL1*β (Figure 4.4). On the other hand, we did observe a dramatic increase in liver TNAP activity (Figure 4.5 A, CKD stool 5,104 mU/mg protein vs non-CKD stool 863.4 mU/mg protein, $P<0.05$) and L-phenylalanine sensitive ALP activity (Figure 4.5 B, CKD stool 1,686 mU/mg protein vs non-CKD stool 161 mU/mg protein, $P<0.05$) in mice receiving CKD stool. Furthermore, mice receiving CKD stool had a 2-fold increase in hepatic gene expression of TNAP (Figure 4.6, $P=0.06$). Immunohistochemistry using an IAP antibody revealed increased hepatocyte staining around portal triads and central veins in mice receiving CKD stool versus non-CKD stool (Figure 4.7 A). Mice receiving non-CKD stool had largely hepatocyte nuclear staining, along with a distinct pattern of staining around the portal triads (Figure 4.7 B). Lastly, I.P. injection of LPS into wild-type mice resulted in reduced liver TNAP activity (Figure 4.8, LPS 1,202 mU/mg protein vs vehicle 1,908 mU/mg protein, $P<0.01$).

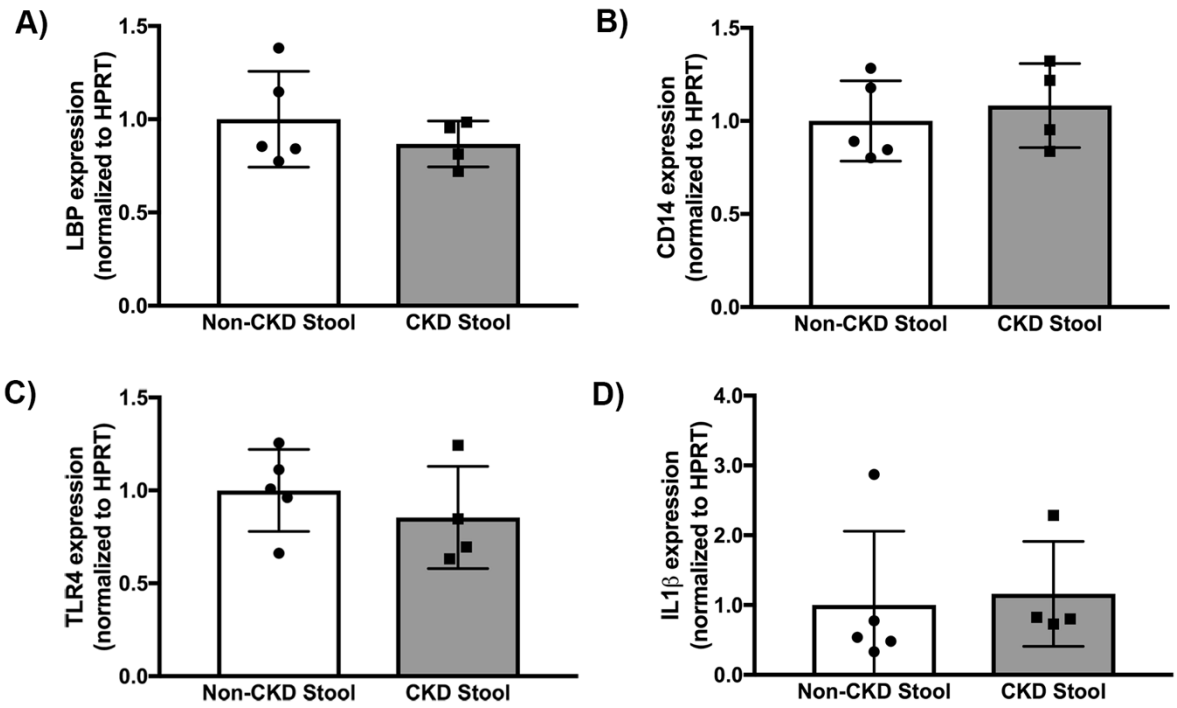

Figure 4.4 Quantitative-PCR analyses of inflammatory markers in the liver.

Comparing wild-type mice that received CKD stool to those that received non-CKD stool, there was no change in hepatic gene expression of **(A)** *LBP*, **(B)** *CD14*, **(C)** *TLR4*, and **(D)** *IL1β*.

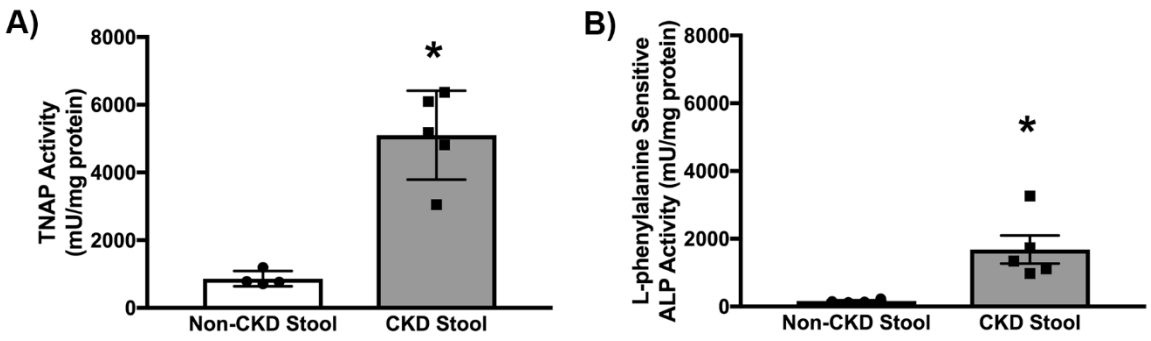

Figure 4.5 Liver ALP activity after fecal transplant. There was an increase in liver **(A)** TNAP and **(B)** L-phenylalanine sensitive ALP activity in wild-type mice receiving CKD stool versus non-CKD stool. *P<0.05 vs non-CKD stool controls

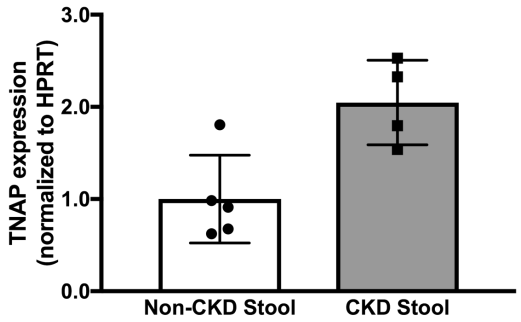

Figure 4.6 Hepatic TNAP gene expression after fecal transplant. There was an increase in TNAP gene expression in mice receiving CKD stool compared to those that received non-CKD stool (P=0.06).

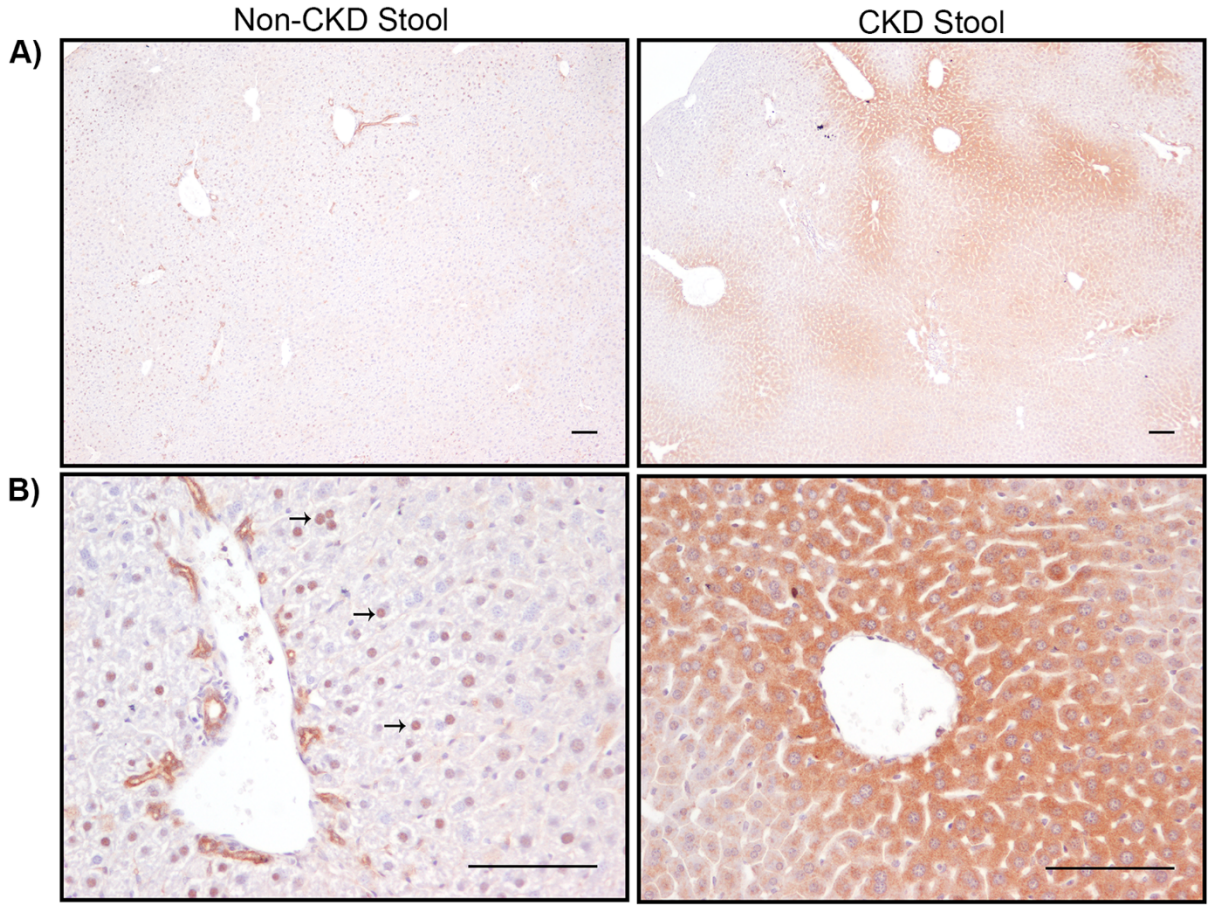

Figure 4.7 Liver immunohistochemistry staining with IAP antibody. (A) We observed increased IAP staining in livers of wild-type mice that received CKD stool compared to those that received non-CKD stool (scale bar = 100 μm, 40X magnification). **(B)** Under higher magnification, there was nuclear staining (arrows) and a distinct staining pattern around the portal triads in the livers of mice that received non-CKD stool. On the other hand, mice that received CKD stool demonstrated predominantly cytoplasmic IAP staining of hepatocytes around portal triads and central veins (scale bar = 100 μm, 200X magnification).

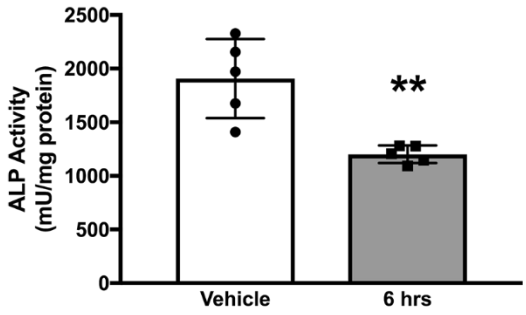

Figure 4.8 Liver ALP activity after LPS injection. There was a decrease in ALP activity 6 hours after IP injection of LPS compared to mice receiving vehicle. **P<0.01 vs vehicle

DISCUSSION

Defects in intestinal ALP activity are implicated in the pathophysiology of several diseases characterized by intestinal barrier defects, while alterations to liver ALP activity are well-documented in a number of hepatobiliary diseases. Despite these observations, studies elucidating factors regulating ALP enzymatic activity are sparse. Taking into account the limited knowledge regarding the regulation of ALP activity, and the intimate link between the intestinal microbiota, the intestine, and the liver; we hypothesized shifts in the microbiota composition induced by CKD may regulate the intestinal and liver ALP activity we observed in Chapter 3.

Despite observing no change in duodenum IAP activity in wild-type mice receiving CKD stool versus non-CKD stool, we observed increased whole duodenum IAP protein expression that accompanied a more intense cytoplasmic staining of enterocytes in the mice receiving the CKD stool (Figure 4.3). Taken together, these findings of higher IAP protein expression with no apparent change in enzyme activity would imply less enzymatic efficiency of the duodenum IAP protein expressed in mice treated with CKD stool. One possible explanation for this finding could be that gavaged CKD stool possessed more IAP protein that may regulate host IAP expression. However, immunoblotting of the cecal content administered to these animals showed no detectable IAP expression, and these mice were sacrificed one week after the last treatment, likely allowing for sufficient clearance of any exogenous IAP protein that was delivered to these animals. In current studies, we are actively treating mice with oral IAP therapy and plan to examine the effect of exogenous IAP treatment on native duodenum IAP activity and protein expression.

An alternative explanation for these previous observations is that CKD stool may have promoted an environment that results in decreased IAP enzyme efficiency. Based on the current state of knowledge, plausible biological changes that could reduce IAP enzyme activity could be increases in the intestinal phosphate content (159), disruption of the luminal pH, or depletion of zinc and magnesium ions required for optimal IAP activity. Regardless of the cause, inefficient IAP enzymatic activity may stimulate a response from enterocytes in the recipient host to upregulate IAP protein production; therefore, compensating for below average enzymatic activity that then results in equal total activity levels. If such a scenario were true, it would be interesting to examine native IAP expression and activity in *Col4a3*$^{-/-}$ (CKD) mice at an earlier time point to determine if IAP expression patterns of wild-type mice treated with CKD stool are replicated at earlier time points of CKD progression in *Col4a3*$^{-/-}$ mice. Additionally, to determine whether CKD mice have this theoretical compensatory mechanism, we could gavage CKD mice with CKD stool and measure IAP activity and protein expression. If this mechanism is lost in the CKD recipient mice, we could explore this loss of response as a possible therapeutic target.

A final potential explanation for the observed discrepancy between duodenal IAP protein content and enzyme activity in CKD stool-treated mice is that this may be a unique response that results from prior depletion of the intestinal microbiota. The effect of antibiotic therapy on IAP activity in mammals is unknown; hence, the response of IAP activity to colonization is unknown. In the future, we could address this possibility by measuring IAP activity and protein expression after antibiotic therapy, as well as

including a group administered only PBS to explore the effect of natural re-colonization on IAP activity.

Future *in vitro* studies will allow us to delve further into the regulation of IAP activity. As an initial step to better understand the regulation of IAP activity, we could utilize a cell culture model of enterocyte differentiation, in which sodium butyrate is applied to HT-29 that results in the transactivation of the IAP gene (160,161). This system would allow us to explore whether a circulating factor increased in the serum of CKD patients may regulate IAP activity. If culturing these cells with uremic serum suggested a circulating factor was indeed present, we could use this system to further explore the contribution of individual uremic toxins, especially those of intestinal origin. In addition to uremic toxins, CKD patients have increased circulating levels of the secondary bile acid deoxycholic acid (DCA) compared to non-CKD controls (162). DCA is generated in the intestinal lumen by the bacterial metabolism of the primary bile acid, cholic acid. If we observed DCA to diminish IAP activity *in vitro*, we could design *in vivo* experiments in which we orally administer DCA to CKD mice.

Our other notable finding in these fecal transplant experiments was a significant increase in hepatic ALP activity in the wild-type mice receiving CKD stool compared to those receiving non-CKD stool (Figure 4.5). These increases in ALP activity was accompanied by increased TNAP gene expression, and increased IHC hepatic staining with the IAP antibody. Interestingly, the mice receiving the non-CKD stool had a distinct staining pattern around portal triads, and IAP nuclear staining was a common observation. While we speculated that this increased liver ALP activity may be due to increased bacterial translocation in mice receiving CKD stool, we did not observe an

increase in the expression of inflammatory markers in the liver (Figure 4.4) that would be expected to accompany endotoxin migration into the portal circulation. Additionally, IP injection of LPS resulted in decreased hepatic ALP activity, possibly due to the production of inflammatory cytokines which are known to inhibit the activity of other ALP isoforms (155). Another possible regulator induced by fecal transplant may be changes in bile acid concentration or metabolism. Limited studies do suggest altered bile acid production and transport in CKD animal models (163). A simple first step to test this possibility could be to stain livers for the farnesoid X receptor (FXR), a bile acid sensor, to determine if we see co-localization with areas of IAP staining and activity.

As with the intestinal ALP activity, *in vitro* studies are necessary to delve into the mechanisms inducing hepatic ALP activity in the setting of kidney injury. We would use a similar approach as described above. First, determine whether a circulating factor in the serum of CKD patients or animals can increase hepatic ALP activity. If so, we would then examine the effect of uremic toxins on ALP activity. Any uremic toxin that may result in upregulation of liver ALP activity can then be injected into mice to determine whether a similar response is induced *in vivo*.

To explore the translational aspect of our findings, we could produce ‘humanized’ mice, as previously described (164,165). In such a study, fecal samples from non-CKD and CKD patients would be used to perform fecal transplant studies in mice. However, one major difference with these studies would be the origin of the fecal content, as human stool would be colon origin, whereas our described studies in mice utilized cecal stool samples. Stool from these two sites would not only be expected to differ in the microbial composition, but would likely also contain numerous other substances specific

to the intestinal origin of the fecal contents. Moreover, since the intestinal microbiota composition of mice and humans varies drastically (166), it is unclear if even human cecal contents could elicit a similar response to that from mice.

The current work has both strengths and weaknesses. The most notable strength is that this is the first study to examine whether the intestinal microbiota of CKD animals can transfer a pathological phenotype and regulate ALP activity. A significant limitation of our studies is a lack of understanding of the mechanisms driving the observed changes in ALP activity. We plan to sequence the recipient mouse stool samples to determine whether the intestinal microbiota composition differs between mice treated with CKD versus non-CKD stool. This analysis could provide additional data to support a role of the microbial composition in regulating these ALP responses and possibly gut barrier function. As we have obtained intriguing results with this initial study, we will need to design more rigorous future studies which will test the effect of antibiotic therapy itself on ALP activity, and possibly include a cohort of mice that receive vehicle treatment alone (PBS) to determine the effect of natural re-colonization of the gut on ALP activity. Lastly, it will be important to repeat these experiments with stool collected from other animals to ensure the observed effect is not limited to just the individual mice used to make these fecal slurries.

In summary, this is the first study to show evidence that transplant of a CKD intestinal microbiota can regulate ALP activity within the intestine and the liver. If future studies confirm the clinical significance of IAP in regulating the intestinal barrier in CKD, these studies will provide valuable mechanistic insight into the regulation of duodenum

IAP and liver ALP activity. This information could aid in the design of effective therapeutic targets.

CHAPTER 5: DISCUSSION

TMAO Summary

In this dissertation, I have provided evidence in an adenine-induced model of CKD that the accumulation of circulating TMAO may be partially related to increased hepatic generation of this compound through increased FMO activity. Our data, combined with prior evidence suggesting that shifts in the intestinal microbiota may also contribute to increased production of TMAO in CKD (118), supports the notion that high circulating TMAO concentrations that accompany CKD progression may not be fully attributable to dysfunctional renal clearance of TMAO. If future studies continue to support TMAO as a direct contributor to cardiovascular disease in CKD, our findings provide evidence for hepatic FMO activity as a potential target for lowering serum TMAO concentrations. Such therapies could be especially beneficial in ESRD patients, as this population frequently exhibits TMAO levels that are 30 to 100-fold higher than individuals with intact kidney function.

TMAO Limitations

While there is early evidence for a correlation between TMAO and adverse CVD events in CKD patients, evidence that reducing TMAO concentrations alters CVD outcomes in this population has not yet been demonstrated. Since CKD is associated with the accumulation of an array of uremic toxins, it will be important to conduct future animal studies to determine whether TMAO is truly involved in CVD progression. Such experiments would be crucial to understanding whether TMAO is truly a modifiable CVD risk factor that is worthy of targeting with future therapeutics aimed at reducing CVD burden in patients with CKD. In addition to the effect of TMAO directly on CVD

progression, our studies described in Chapter 2 neglected to investigate the mechanisms contributing to increased hepatic FMO activity in CKD.

TMAO Future Work

Translational experiments in CKD mice aimed at evaluating the direct contribution of TMAO to CVD progression would help provide critical justification for the potential clinical significance of TMAO elevations to adverse cardiovascular outcomes in humans with CKD. Prior studies suggest that kidney disease exacerbates atherosclerosis development in the *ApoE*$^{-/-}$ mouse model of atherosclerosis (167) and results in the development of cardiac hypertrophy (168). As TMAO is implicated in the development of both of these diseases in non-CKD models, it would be of interest to determine if reductions in TMAO in *ApoE*$^{-/-}$ mice with CKD alters these cardiovascular endpoints. As other gut-derived toxins besides TMAO have known pathological effects on the cardiovascular system, therapies targeting solely TMAO generation will be necessary to determine the specific contribution of TMAO to disease development. Targeting gut microbes with broad spectrum antibiotic treatment would likely result in the lowering of multiple gut-derived uremic toxins; thus, studies could be designed using compounds known to specifically inhibit the bacterial enzymes involved in TMA generation from its dietary precursors, choline and L-carnitine. One such example is 3,3-dimethyl-1-butanol (DMB), a compound that inhibits the bacterial conversion of choline to TMA (120).

The most noteworthy finding of our study was that induction of kidney disease in both male and female mice led to increased hepatic FMO activity. This finding generates several interesting future research directions: (1) to determine the factors

driving increased FMO activity in CKD, and (2) to explore how targeting FMO activity in CKD mice alters serum TMAO concentrations and clinical outcomes (i.e. survival, CKD progression, cardiac hypertrophy). As discussed in chapter 2, the accumulation of urea in CKD may be one possible driver of increased FMO activity. An alternative hypothesis may be that increased TMA generation resulting in increased substrate availability may stimulate hepatic FMO3 expression and activity. To address this possibility, we can assess FMO3 expression and activity in the wild-type mice that received stool content collected from non-CKD and CKD mice as described in Chapter 4. If a factor from the CKD microbiota is regulating hepatic FMO3 expression, we would expect the wild-type mice that received stool collected from CKD mice to have increased expression and activity compared to the wild-type mice that received non-CKD stool. If experiments reveal a direct contribution of TMAO to the development of CVD in CKD mouse models, studies could be conducted to determine whether inhibition of hepatic FMO activity (i.e. CKD induction in *FMO3*$^{-/-}$ mice) decreases TMAO accumulation and attenuates CVD development.

Alkaline Phosphatase Summary

In a separate set of studies, we examined the effect of kidney disease on ALP activity within both the intestine and liver. Our first notable finding was that *Col4*α*3*$^{-/-}$ mice, a model of CKD, demonstrated a significant decrease in duodenum IAP enzymatic activity accompanied by altered cellular localization of this protein. Additionally, we have preliminary IAP activity data from a second CKD mouse model (adenine diet), that appears to confirm these findings; however, our protein expression analysis in this model is still ongoing. We speculate that this decreased duodenum IAP

activity may contribute to intestinal barrier dysfunction in CKD. To gain insight on factors regulating IAP activity, we designed a fecal transplant study in which we depleted the microbiome of wild-type mice, then randomized these animals to be recolonized with stool from either non-CKD or CKD mice. We observed no change in duodenum IAP activity between these groups, but did find increased IAP protein expression in duodenal tissue from wild-type mice recolonized with CKD stool compared to those receiving non-CKD stool. While this result was unexpected, this may provide some evidence to suggest regulation of duodenum IAP production by fecal contents. Regardless, based on our consistent finding of decreased duodenal IAP expression and activity in CKD mice, IAP therapy may be a promising strategy for targeting intestinal barrier dysfunction in CKD. In support of this idea, IAP therapy attenuates inflammation in several other disease models exhibiting defects in intestinal barrier dysfunction. Importantly, in both mouse studies and human clinical trials conducted thus far, no major side-effects have been reported with IAP therapy.

In addition to altered duodenum activity, liver ALP was convincingly increased in both the adenine-induced and *Col4α3* mouse models of CKD. Interestingly, in the fecal transplant study, we observed substantially higher liver ALP activity in the wild-type mice receiving CKD stool compared to those that received non-CKD stool. We hypothesize that this upregulation of liver ALP activity in response to fecal transplant with CKD stool may provide evidence that constituents in CKD feces are either: (1) actively inducing gut barrier defects that lead to entry of bacterial toxins into portal blood, or (2) serving as direct regulators of liver ALP activity.

Our current working hypothesis is that either the accumulation of uremic toxins or the physiologic changes that accompany kidney disease progression (i.e. acidosis, mineral metabolism defects) leads to decreased IAP activity. In turn, this lack of IAP activity facilitates a shift in the fecal microbiota composition and defects in intestinal barrier integrity, possibly resulting in increased bacterial byproducts entering the portal circulation to stimulate liver ALP activity (Figure 5.1).

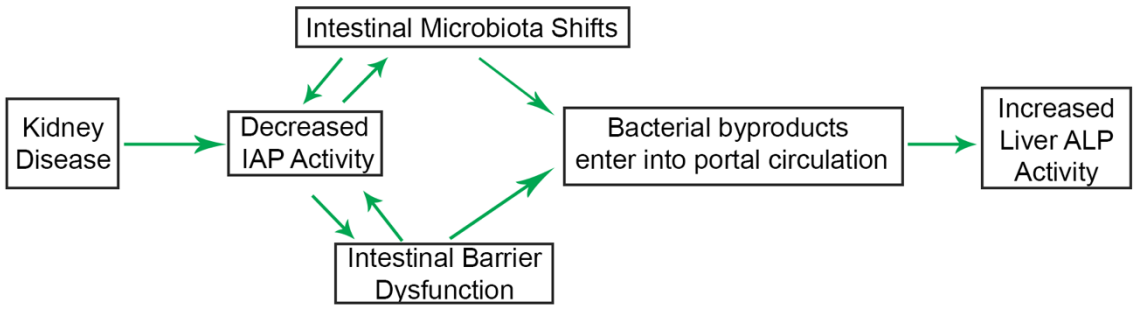

Figure 5.1 Working Hypothesis for ALP Activity Changes in CKD. We hypothesize that the uremic milieu induced by kidney injury suppresses IAP activity, which could lead to both changes in the relative abundance of intestinal bacteria and impaired intestinal barrier function. These pathologic changes could facilitate the entry of bacterial toxins into the portal circulation that stimulates liver ALP activity and inflammation.

Alkaline Phosphatase Limitations

While this dissertation presents strong evidence for diminished duodenum IAP activity in CKD, we are unsure whether decreased IAP activity occurs before or after shifts in the intestinal microbiota and increased intestinal permeability. It is plausible that either uremia-induced microbiota shifts or decreased IAP activity could be early events capable of promoting altered intestinal barrier function in CKD. Furthermore, at the present time, we are unsure whether *Col4α3*$^{-/-}$ mice truly exhibit functional defects of the intestinal barrier, as this has not yet been formally studied. Lastly, the physiological significance of increased liver ALP activity is unknown. As the liver is a major site of bacterial detoxification from portal blood, we speculate that liver ALP activity is involved in this function; yet, further experiments will need to be performed to validate this hypothesis.

Alkaline Phosphatase Future Work

To determine the relationship between duodenum IAP activity and intestinal barrier function, we first need to determine whether there is a defect in intestinal barrier function in the *Col4α3* at 10 weeks of age, the age in which we characterized IAP expression and activity in Chapter 3. To determine this, we can orally challenge *Col4α3* with fluorescently labeled dextran (FD4 MW 3,000-5,000), and measure the appearance of labeled dextran into systemic circulation. Initial studies utilizing dextran of various sizes will be advantageous as the extent of increased intestinal permeability in CKD mice is unknown. If evidence for barrier dysfunction was observed in these mice, we could design longitudinal studies examining the sequential relationship between IAP activity and intestinal barrier function along the timeline of CKD progression.

Additionally, these dextran gavage studies will help determine the size limit of increased intestinal permeability in CKD mice. To determine whether IAP can modulate microbiota composition in CKD mice, we will perform 16S rRNA sequencing of stool content collected from CKD mice randomized to receive either vehicle or IAP by oral gavage.

Concluding Remarks

An ideal therapy to attenuate comorbid disease development in patients with CKD would modulate the inflammatory phenotype and decrease circulating levels of uremic toxins in patients. It may be possible that the IAP therapy could achieve both of these outcomes by shifting intestinal microbiota composition and fortifying the intestinal barrier. In addition to assessing the inflammatory profile of CKD mice treated with IAP, we can measure serum levels of TMAO and other uremic toxins, such as indoxyl sulfate and p-cresyl sulfate. However, our current lack of understanding about the mechanisms contributing to intestinal microenvironment changes in CKD make it difficult to design targeted therapies at this time.

Several important issues need to be addressed to move the field of gut biology in CKD forward. First, there is still no solid published evidence that humans or animal models of CKD exhibit functional defects in the intestinal barrier. To date, the vast majority of publications merely speculate altered function based on changes in barrier protein expression patterns or evidence of a bacterial presence in the systemic circulation. Moreover, designing *in vivo* studies to test intestinal barrier function is not a simple task, as studies evaluating the emergence of labeled substances in the bloodstream following an oral gavage may be confounded by reduced elimination of these substances in the setting of kidney disease. Second, detailed investigations are

required to determine whether an accumulation of gut-derived uremic toxins is due to reduced renal clearance or shifts in the intestinal microbiota that results in increased capacity to generate the toxin. With advances in metagenomic sequencing, it is now possible to determine if specific bacterial genes which contribute to toxin production may be altered in bacterial populations harvested from CKD patient stool. This is a far more informative technique compared to traditional 16S rRNA sequencing that only provides data on relative bacterial abundance in a given sample. Lastly, since the majority of stool samples collected from patients merely reflect the bacterial populations present in the colon, it will be imperative to conduct future studies aimed at identifying bacterial changes that occur in other intestinal segments in CKD, particularly the small intestine. Due to difficulties with conducting such studies in humans, translational experiments in CKD mouse models are an important first step for guiding these future investigations.

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